BACTERIAL GENETICS

BRAUN

CFTRI-MYSORE
2921
Bacterial geneti..

2921

1. bacterial genetics
2. Mutations
3. Mutagenic agents
4. Mutagenesis
5. Mutants
6. population changes
7. bacterial recombination
8. " transformation
9. Bacterial transduction
10. bacteria

BACTERIAL GENETICS

WERNER BRAUN, Ph.D.

ILLUSTRATED

W. B. SAUNDERS COMPANY
Philadelphia 1953 London

2921

GV, 21;6
N53

Copyright, 1953, by W. B. Saunders Company

COPYRIGHT UNDER THE INTERNATIONAL COPYRIGHT UNION

All rights reserved. This book is protected by copyright. No part of it may be duplicated in any manner without written permission from the publisher.

Made in the United States of America
Press of W. B. Saunders Company, Philadelphia

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 53–6639

CFTRI-MYSORE
2921
Bacterial geneti.

Preface

THE HISTORY of science is characterized by sudden spurts of progress which frequently occur following the merger of once separated disciplines. The field of bacterial genetics represents one of the most recent examples of such scientific cross-fertilization from which a subject with real hybrid vigor has evolved. The progress in this field during the last ten years has contributed not only significant information for geneticists, but what is perhaps even more important, it has resulted in the development of a branch of science that may take its place alongside biochemistry as necessary for understanding the nature and activity of microorganisms. Thus it has been recognized that a majority of problems in bacteriology and its allied fields, such as medicine, public health, bacterial metabolism and industrial fermentation, require an understanding of the nature and control of variation. This recognition of the growing importance of bacterial genetics is reflected in the remarkable increase of bacteriological publications that either deal with genetic problems or utilize the techniques developed by bacterial geneticists. In addition several universities have added formal courses on the subject to their curriculum.

Yet, despite the fact that bacterial genetics is gaining such importance in basic and applied research, it also has become evident that many students and professional workers without specialized training in genetics have had difficulties in understanding and following pertinent progress in this field. Apparently, there are two primary reasons for these difficulties: (1) the geneticists who work with bacteria have transferred many of their special terms into the field of bacterial genetics, and much of this language, as well as many of the underlying principles, are foreign to students of bacteriology; (2) the places of publication of fundamental data have been widely scattered, many of the important papers hav-

ing appeared in journals that are read by very few bacteriologists.

This book has been written in an attempt to aid in overcoming these difficulties. It tries to present the more important findings and principles of bacterial genetics to those primarily trained, or being trained, in bacteriology, and it tries to provide a basis for better evaluation of the relationship between bacterial genetics and such diverse problems as pathogenesis, general laboratory techniques, bacterial physiology, epidemiology, therapy, and taxonomy. Beginning with a review of those general principles of genetics which are necessary for the understanding of bacterial genetics, the text endeavours to furnish a general account of the present status of bacterial genetics in a manner which, it is hoped, is elementary enough for the beginning student and sufficiently comprehensive to be of value for the research worker. But even though this book was written with the needs of bacteriologists in mind, it is hoped also that its contents will prove equally useful to students and research workers in the general field of genetics. The latter may find here not only a compilation of progress in an allied field but also information that should be of value to basic genetic problems.

In presenting this outline of bacterial genetics no attempt has been made to cover all the details of this rapidly expanding field and the space alloted to the various problems has been determined to a large extent by the apparent importance of the particular problem for general bacteriological questions. Thus the relatively extensive discussion of population changes should not be blamed on the author's preoccupation with such problems but on his conviction that these problems must be thoroughly understood and recognized by anyone connected with studies on bacteria. Throughout the book only representative examples of published data are cited and consequently many equally valuable contributions to the subject had to be omitted both in the text and in the literature references. In most instances, an effort has been made to cite those literature references which in turn will guide the reader to older publications.

I am greatly indebted to many colleagues and friends for their assistance in the preparation of this book. Many of them have contributed illustrations, including some previously unpublished material. The names of these contributors are indicated in the figure legends. I wish to express my collective thanks to them as well as

to the various publishers who gave permission for reproducing previously published figures and tables. I am particularly indebted to Drs. Emily Kelly, Edward Garber, Vernon Bryson and Mr. Leonard Mika, who read the manuscript in its entirety, and to Drs. Joshua Lederberg, Rollin Hotchkiss and Robert Austrian, who read certain chapters of the manuscript. All of them contributed valuable suggestions and editorial advice but, of course, they are in no way responsible for any remaining errors or omissions. The artistic skill of Mr. James Decker, who prepared all of the original drawings, and the diligence of Mr. Austin Hoffman, who typed the entire manuscript, were of invaluable help in the preparation of the book. I also wish to express my sincere appreciation to the staff of W. B. Saunders Co.; their interest and efficiency were of great help at all times. Finally, it is a real pleasure to acknowledge the interest of Dr. William Burrows without whose encouragement this book probably would still be an idea in the author's mind.

W.B.

Thurmont, Md.
July, 1953

Contents

Chapter 1

SOME GENERAL GENETIC PRINCIPLES 1

The nature of hereditary characteristics 2
Genotype and phenotype 2
The site and transmission of hereditary determinants 4
Chromosomes and genes 4
Chromosome numbers and mitosis 5
Meiosis 6
Polyploidy 9
Dominance 9
Linkage and segregation 11
The stability of hereditary determinants 12
Mutation 12
Mutagens 14
Chromosomal changes 14
Gene action 15
Extra-nuclear determinants of hereditary characteristics 20
Cytoplasmic inheritance in plants and *Drosophila* 20
The killer characteristic of *Paramecium* 21
Antigenic transformation in *Paramecium* 23
Cytoplasmic inheritance in yeast 25
Interactions between nuclear and cytoplasmic elements . 25
Enzymatic adaptation 26
Population changes 28
Conclusions 29

Chapter 2

THE EVOLUTION OF BACTERIAL GENETICS 32

Adaptation 33

Life-cycles 35
Mutation and selection 36

Chapter 3

THE CYTOLOGICAL BASIS OF BACTERIAL GENETICS .. 38

Nuclear bodies 38
Intranuclear structures 44
Reproductive mechanisms 46
Bacterial cytogenetics 48

Chapter 4

MUTATION 49

Evidence for the occurrence of mutations in bacteria 50
Newcombe's spreading experiment 50
The fluctuation test 52
Lederberg's indirect selection test 55
Studies on the mutational origin of tryptophan-independence 58
Generality of phenomenon 60
Mutation rates 62
Methods for estimating mutation rates 62
Mutation rate vs. mutant frequency 66
Mutation rate and cell division 67
The cause of spontaneous mutations 67

Chapter 5

MUTAGENIC AGENTS AND MUTAGENESIS 69

Dosage effects 70
Lethal effects and mutation rates 73
Determination of induced mutation rates 73
Phenomic lag 73
Nuclear structures as sites of mutational changes 76
Mutational lag 77
"Zero point" and "delayed" mutants 79
Photoreactivation 79

Chapter 6

REPRESENTATIVE TYPES OF MUTANTS. I 81

Mutants resistant to inhibitory agents 81
Streptomycin resistance 81
Penicillin resistance 82
Isolation of antibiotic-resistant mutants 83
Cross resistance and collateral sensitivity 85
Non-mutational increases in antibiotic resistance 87
Antibiotic resistance in relation to therapy 87
Radiation resistance 88
Biochemical mutants 90
AUXOTROPHIC MUTANTS 90
Isolation procedures 91
Assaying growth requirements 94
Polyauxotrophic strains 95
Reverse mutations 95
Blocking of biosynthetic steps and syntrophism 97
Interactions between different biosynthetic pathways ... 98
Some applications of auxotrophic mutants 99
FERMENTATION MUTANTS 100
PIGMENTATION MUTANTS 103

Chapter 7

REPRESENTATIVE TYPES OF MUTANTS. II. 104

Mutations affecting colonial morphology 104
Detection 104
Colony types 107
The significance of changes in colonial morphology 108
Colonial morphology as indicator of changes in resistance and growth-requirements 109
Environmental effects upon colonial morphology 112
Antigenic mutants 114
Antigenic types 114
The cause of antigenic changes 115
Phenotypic modifications 115
Variation in antigenic complexes 116
Phase variation in *Salmonella* 117
Simple indicators of antigenic changes 118
Reversions 120

Mutants with altered virulence 121
Inherent properties affecting virulence 121
Associated changes 123
Some genetic problems common to colonial, antigenic and virulence mutants 124
Mutation rates 124
Nuclear or cytoplasmic factors 126
Antigenicity and virulence of auxotrophic mutants 127
Independent and associated changes in colonial, antigenic, virulence and other characteristics 133

Chapter 8

POPULATION CHANGES DUE TO MUTATION AND SELECTION. I 138

POPULATION CHANGES DURING GROWTH UNDER RELATIVELY CONSTANT ENVIRONMENTAL CONDITIONS 139
Population equilibria between mutants of equal growth rate 139
"Periodic selection" 141
POPULATION CHANGES IN THE ABSENCE OF CONSTANT ENVIRONMENTAL CONDITIONS 144
Absolutely selective conditions 144
Relatively selective conditions 145
POPULATION CHANGES ON SOLID MEDIA 145
POPULATION CHANGES IN LIQUID MEDIA 148
Typical growth cycle 148
Population pressure 149
Factors influencing rate of population changes 150
The role of metabolites in population changes 151
Apparent cycles and reverse population changes 154
The effect of the source of N upon population changes .. 155
Additional correlations between metabolite accumulation and population changes 156
Other environmental agents affecting population changes 158
Correlations between population changes and inherent differences in metabolism 159
Interactions between mutants in mixed populations 159
Population changes involving inhibitory effects produced by the parent type upon mutant cells 163
Determination of the selective value of a mutant 164

Chapter 9

POPULATION CHANGES DUE TO MUTATION AND SELECTION. II 165

Population changes IN VIVO 165
Examples of population changes IN VIVO 165
The nature of selective host factors 167
The effect of amino acids upon population changes IN VIVO 168
Mutation and selection in relation to some problems of bacteriological technique 170
Maintenance of bacterial stock cultures 170
Preparation of inocula 172
Problems arising during cultural growth 173
Primary isolations 174

Chapter 10

POPULATION CHANGES THAT ARE NOT DUE TO MUTATION AND SELECTION 176

Modifications 176
Enzymatic adaptation 178
Manifestations and detection 178
Genetic control of enzymatic adaptation 179
Simultaneous adaptation 180
Kinetics of enzymatic adaptation 181

Chapter 11

RECOMBINATION IN BACTERIA 184

Mechanism of crossing-over in higher organisms 184
Recombination in *E. coli*, K-12 186
True recombination versus transformation 191
Mechanism of bacterial recombination 192
Prevalence of recombination among different bacteria .. 194
Problems that require recombination for testing 194

Chapter 12

BACTERIAL TRANSFORMATION AND TRANSDUCTION . 197

Transformation 197
Definition of transformation 197
Transformation of pneumococcal capsular types 198
Pneumococcal transformations involving intermediate capsular types 201
Transformation reactions involving protein specificity, drug resistance and colonial morphology 204
Cellular competence and environmental conditions required for transformation 207
Time required for action of DNA 210
Mechanism of bacterial transformation 211
Transduction in Salmonella 212
General importance to other problems 215

Chapter 13

BACTERIAL GENETICS IN RELATION TO GENERAL BACTERIOLOGICAL PROBLEMS 216

Metabolism 216
Pathogenesis and epidemiology 218
Taxonomy 223
Evolution 225

INDEX 229

CHAPTER 1

Some General Genetic Principles

More than fifty years of genetic research on higher organisms have demonstrated that, except for minor peculiarities in some species, the basic principles of genetics, the science of heredity, apply equally to all animals and plants. Information obtained in experimental work with the fruit fly, *Drosophila,* or with corn, therefore, can be and has been applied, often with immense economic gain, to such diverse problems as improvement of crops and livestock, increased penicillin yields, the discovery and understanding of the Rh factor, and the determination of disputed paternity. In recent years the area of applicability of general genetic principles has been broadened by the demonstration that the general mechanisms of inheritance in unicellular organisms, including bacteria, do not differ significantly from those established for higher organisms. Even though in most cases reproductive mechanisms appear to be less complex in bacteria than in higher organisms, all the available evidence indicates that the determinants of hereditary characteristics, their nature, sites, transmission, stability and the manner in which they control the processes leading to the actual development of the detectable characteristics, are similar in bacteria and in higher organisms. During its rapid development the field of bacterial genetics, therefore, was able to draw on the vast amount of existent general genetic information, the type of information which will be reviewed briefly in the following paragraphs.[1]

[1] This introductory survey is intended to acquaint the reader with genetic terminology and those basic genetic principles that are of interest to bacterial genetics. Within the confines of this survey it is impossible to do full justice to the wealth of data accumulated in genetic studies with higher organisms. For a more detailed account the reader is referred to one of the many textbooks of genetics. For example: Sinnot, E. W., Dunn, L. C., and Dobzhansky, Th., *Principles of Genetics,* McGraw-Hill Book Co., 1950; Srb, A. M. and Owen, R. D., *General Genetics,* W. H. Freeman & Co., San Francisco, 1952; Dunn, L. C. (Editor), *Genetics in the 20th Century,* The Macmillan Co., New York, 1951.

THE NATURE OF HEREDITARY CHARACTERISTICS

Genotype and phenotype

The old saying "like begets like" expresses the well-known fact that offspring or *progeny* usually mirror the general characteristics of their parents. This applies not only to higher organisms, where sexual processes are the rule rather than the exception, but also to asexually reproducing organisms. Hereditary traits thus are transmitted from generation to generation, and the manner in which they are transmitted has been the subject of intense experimental research, starting with the ingenious experiments on peas conducted by the Austrian monk, Gregor Mendel, in the 1860's. These studies, initially unnoticed, were independently rediscovered and extended by DeVries, Correns and Tschermak in 1900 and since then research has continued on various animals and plants in laboratories throughout the world. These extensive investigations on the principles of heredity and variation have demonstrated that what is transmitted from generation to generation is a set of determinants of as yet ill-defined chemical nature, controlling the reaction range of an individual or a cell. The sum total of these heritable determinants is called the *genotype*. Everyday experience shows us that despite similarity in genotype, as, for example, in identical twins or a group of purebred plants maintained under different environmental conditions, considerable differences may exist in appearance, i.e., the manner in which the inherited traits express themselves under the influence of the environment. What is inherited therefore is an ability to react in a specific way to specific environmental conditions. The sum total of the realized characteristics, in other words, the appearance of morphological characteristics as well as the manifestation of physiological processes in individuals, is called the *phenotype*. Phenotypic differences between organisms of similar genotype are referred to as *modifications*. Thus the interaction between hereditary determinants and environmental influences may cause divergent phenotypes despite the presence of identical genotypes; however, the genotype controls the potential range of such phenotypic differences. To choose a simple illustration let us consider the skin pigmentation of whites and Negroes. This difference in pigmentation is controlled by hereditary determinants *(genotypic differences)* yet the actual skin color will greatly depend on the extent of the individual's exposure to sunlight *(phenotypic differences)*. With prolonged exposure to sunlight, the skin of

whites may become as dark as that of Negroes who have not been similarly exposed; yet after equally intense exposure the latter would manifest a far darker skin pigmentation than that ever possible for a white person (Fig. 1).

The foregoing example illustrates not only the potential plasticity existing in each organism or cell, but it also demonstrates that under certain environmental conditions the phenotypic expression of one genotype (the sum of inherited determinants) may be identical to that of an entirely different genotype. Such a phenomenon has been termed *phenocopy*.

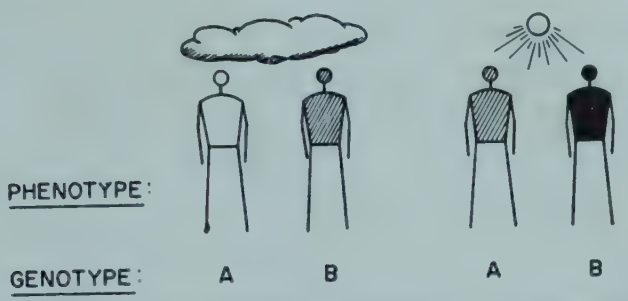


Fig. 1. Sketch illustrating the difference between phenotype and genotype (see text for a description of this example).

Even though environmental effects are capable of modifying the phenotype, as a rule such *modifications* persist only in the presence of the specific environmental condition and will not be transmitted to and inherited by the offspring. Cutting off the tails of mice for many generations does not reduce the length of tails in the progeny; neither does the exposure of flagellated bacteria to phenol, interfering with the development of flagella, cause the disappearance of flagella in the progeny when the latter are grown in phenol-free media. These and many other experimental studies have led to the conclusion that acquired characteristics probably are never inherited. This does not mean that the genotype remains entirely unaffected by the environment. As will be discussed subsequently, changes in the genotype occur occasionally in all organisms, and certain environmental agents are capable of enhancing the rate of such changes. In addition, the environment plays a most important role in promoting the selective survival of individuals with hereditary determinants that control characteristics best adapted to the existing environmental conditions.

It is now well established that practically all characteristics, both

morphologic and physiologic, are under the direct or indirect control of hereditary determinants. In general, it is not the trait itself which is inherited, rather it is the potential to develop the particular trait under proper environmental conditions that is transmitted from generation to generation. Thus learning is not inherited, but the ability to learn is; cancer is not inherited but the tendency to develop certain types of cancer appears to be a hereditary trait. In the final analysis all hereditary characteristics are probably the end products of biochemical processes. This applies equally to physiological characteristics, morphological characteristics, and even to the ability to learn which depends on the development of the brain cells, a process involving an as yet unknown series of complex biosynthetic interactions. Therefore, the hereditary determinants may be said to control in a specific manner biosynthetic reactions and the biochemical activities of an organism. We shall discuss such mechanisms of control in more detail after a brief inquiry into the site of the hereditary determinants and their transmission.

THE SITE AND TRANSMISSION OF HEREDITARY DETERMINANTS

Chromosomes and genes

Most hereditary determinants are transmitted from generation to generation via nuclear components. Within the nucleus of cells there exist microscopically visible structures, usually filamentous in appearance, which are most easily observed when the nucleus is in the process of dividing. Due to their capacity to take up basic dyes these structures are called *chromosomes*. Chromosomes consist of nucleoproteins, i.e., proteins plus desoxyribose nucleic acid (DNA), and exhaustive experimental evidence has established that they are the carriers of factors or chemical complexes that control the inherited traits. These factors within the chromosomes are called genes; they are believed to represent macromolecules which are arranged in a linear fashion. While chromosomes are easily visible, the genes, or what is believed to be their site, have been only approximately localized. For example, the chromosomes of the salivary glands of insects are exceptionally large and, when stained with basic dyes, display a substructure consisting of bands with varying affinities for the stain. Certain alterations in hereditary traits have been found to be associated with changes in this visible substructure of salivary gland chromosomes (see also p. 14 and p. 186). These observations are merely one example of the correlation of

cytological observations with the manifestations of genetically controlled processes, a correlation which has been of immense value in modern genetic research.

Chromosome numbers and mitosis

It was stressed above that as a rule the genotype, or the sum total of hereditary determinants, remains constant from generation to generation, as well as from cell to cell. Since the chromosomes have been recognized as the carriers of these determinants it must be expected that a mechanism exists whereby each cell receives from its parent cell a similar complement of chromosomal, and thus genic, material. Cytological observations have proven that this is the case, and that the manner in which chromosomes are distributed

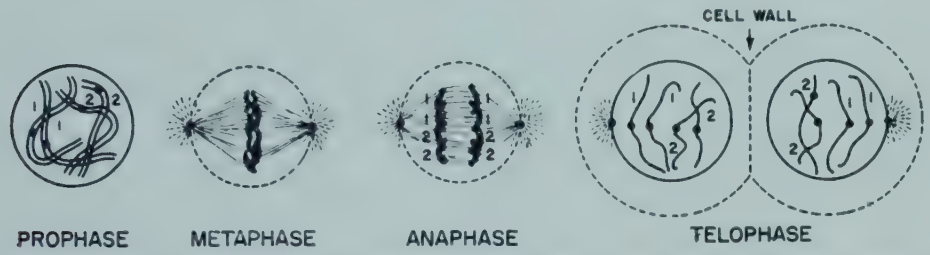


Fig. 2. Diagram of several stages of mitosis. Each cell in above sketch is diploid having 2 pairs of chromosomes. The dark dot on each chromosome, also shown in Figs. 3 and 57, indicates the centromere, a fixed structure which appears directly concerned with the movements of the chromosomes during division, leading the way on the spindle towards the poles.

from generation to generation, as well as from cell to cell, parallels the manner in which hereditary traits are transmitted. First of all it has been demonstrated that within a given species each nucleus contains a constant and characteristic number of chromosomes. For example, man has 24 pairs in each nucleus, *Drosophila melanogaster* 4 pairs, and certain roundworms have 1 pair. Considerable differences in size and shape are often seen between each pair of chromosomes (Fig. 3), but it is typical for sexually reproducing organisms that each chromosome is matched by a partner, or *homologous chromosome,* identical in size and shape and usually similar in its genic complement. Cells with pairs of chromosomes are called *diploid* cells. One member of each pair originates from the mother, the other from the father. During ordinary cell division equal shares of each chromosome are distributed to the daughter cells in a process known as *mitosis*. Figure 2 illustrates various stages of this

process. Just prior to cell division and nuclear division the nuclear membrane disappears, the chromosomes become visible in stained preparation as threads, and each chromosome shows a lengthwise split into two daughter chromosomes. This is known as the *prophase* stage. Prior to the splitting of each chromosome a duplication of the chromosomes, and with it the production of a duplicate or copy of each gene in the chromosome, must have taken place. This self-duplication or *autocatalytic* process is beyond the limits of visual observation. Nevertheless, the actual splitting of the chromosomes is visible and in the next stage of mitosis, referred to as *metaphase*, the split chromosomes are found in the center of the cell, usually lying in a single plane. In the meantime a spindle-like configuration has appeared in the surrounding cytoplasm, containing spindle fibers which appear to connect the chromosomes with the two poles of the spindle. A stainable granule, referred to as *centriole*, is often found at each pole of the spindle. During the next stage, the *anaphase*, the daughter halves of each chromosome separate and move to opposite poles of the mitotic spindle. The process is completed in the *telophase*, when an identical complement of daughter chromosomes lies at opposite ends of the cell. The mitotic spindle disappears, the chromosomes lose much of their visible differentiation, a nuclear membrane is formed around each group of daughter chromosomes, and a cell wall starts to separate the two daughter cells from each other. Each of these cells now contains the same number of chromosomes and with it the same set of hereditary determinants that existed in the parent cell.

Meiosis

In the process of sexual reproduction an even more complex cycle of chromosome distribution assures the maintenance of a constant number of chromosomes from generation to generation. Sexual reproduction involves the union of two cells known as germ cells or *gametes* (e.g., egg and sperm) and thus entails the combination of two cells into one, in contrast to ordinary cell division, or asexual reproduction, where one cell gives rise to two. If gametes were produced by typical mitosis they would contain the usual diploid complement of chromosomes (each chromosome present as a pair), and following fusion of two gametes the resulting cell, or *zygote*, would contain twice the normal number of chromosomes. Such an increase in the number of chromosomes from generation to

generation is prevented by a special type of cell division which occurs during the maturation of the gametes and reduces their chromosome number to exactly one half of the original number. As a result of these reduction divisions, or *meiosis,* gametes receive only one partner of each chromosome pair. Cells in which each chromosome is represented as a single entity, and not paired, are called *haploid* cells. Gametes thus are haploid and the subsequent fusion

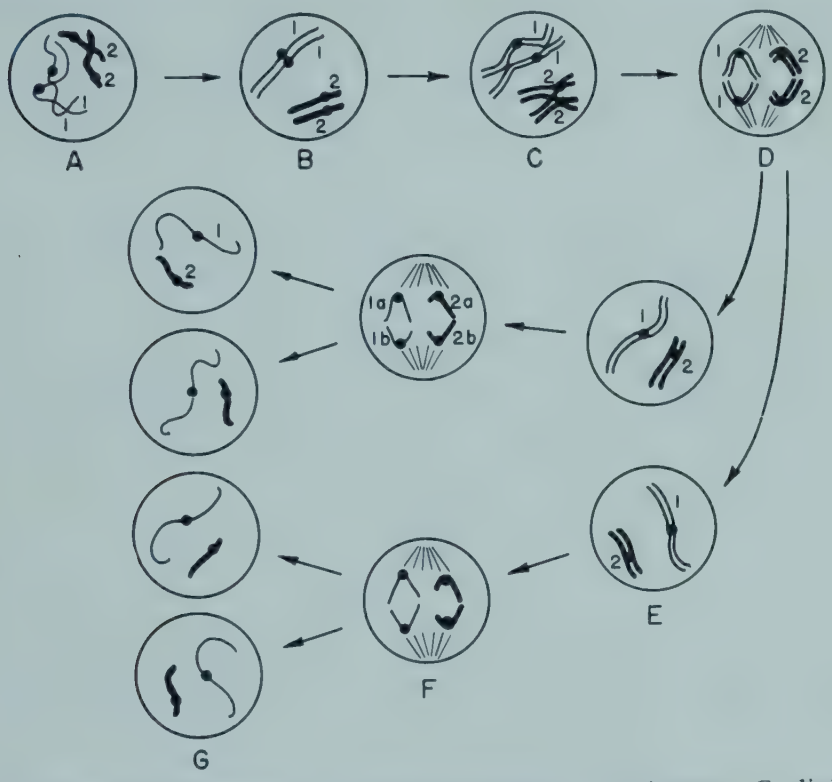


Fig. 3. Diagram of meiosis. *A*, Leptotene stage; *B*, pachytene; *C*, diplotene; *D*, first anaphase; *E*, interphase; *F*, second anaphase; *G*, gametes.

of two gametes restores the diploid condition. Figure 3 shows several stages of meiosis and illustrates the manner in which the duplicate or diploid number of chromosomes is reduced by half. The entire process involves two consecutive cell divisions accompanied by only a single division of the chromosomes. The essential features of meiosis are (1) the intimate pairing of homologous chromosomes during the early stages of the first meiotic division (Fig. 3 B); (2) subsequent lengthwise splitting of each chromosome into two *chromatids* (Fig. 3 C); (3) the separation of homologous chromosomes from each other into two new nuclei and cells, in which each chromosome is present singly but consists of two chromatids (Fig.

3 D-E); and (4) the separation of chromatids from one another during the second meiotic division (Fig. 3 F). The end result is now accomplished, each gamete contains only one partner of the original pair of chromosomes. It will be remembered that the original pair of chromosomes consisted of one chromosome which originated from the father (paternal chromosome) and a homologous chromosome which originated from the mother (maternal chromosome). It is purely a matter of chance whether during the reduction division a gamete receives the paternal or the maternal

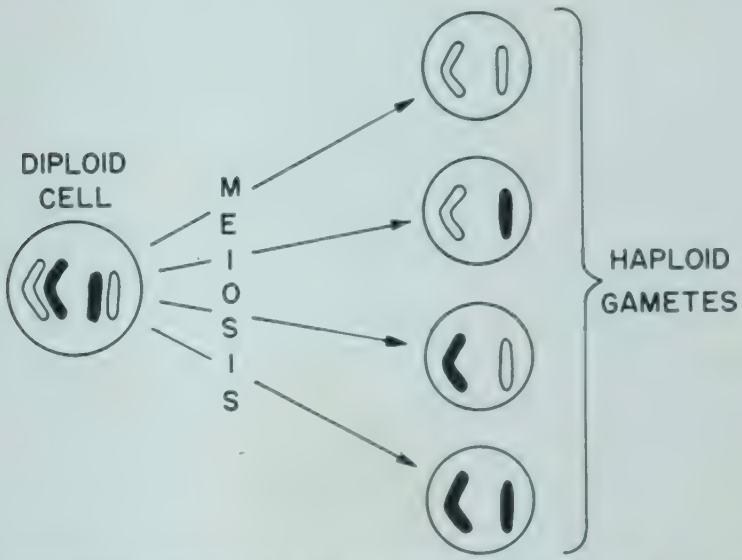


Fig. 4. Diagram illustrating the independent assortment of two pairs of chromosomes during meiosis. The "maternal" chromosome is indicated as white, the "paternal" chromosome as black.

member of the original pair of chromosomes. As a result of this random distribution, the haploid gametes of organisms having more than one chromosome may contain any one of the several possible assortments of maternal and paternal chromosomes (Fig. 4).

The haploid stage is only transient in most sexually reproducing organisms, being restored to diploidy during the subsequent conjugation of gametes. However, asexually reproducing forms, e.g., certain fungi and, as far as we know, most bacteria, can exist in the haploid stage indefinitely, i.e., each chromosome is present as one unit only, reproduces itself and then subdivides lengthwise during cell division (mitosis). Thus the process of mitosis in asexual organisms and the occurrence of meiosis in sexually reproducing forms achieve the same goal: equal chromosome numbers, and with

it similar sets of genetic determinants, are maintained from generation to generation.

Polyploidy

In some species there may be more than two homologous chromosomes in each nucleus. Such polyploids (Fig. 5) may contain three identical sets of each chromosome (triploid), four sets (tetraploid), five sets (pentaploid), or even more than that. Although polyploidy may occur naturally, it can also be induced by certain chemicals, such as colchicine and camphor, that prevent normal splitting of the chromosome during meiosis or mitosis. Polyploids

Fig. 5. Some diagrammatic examples of polyploid cells.

usually have giant cells and the polyploid condition may be maintained indefinitely.

Dominance

Having discussed the chromosomes it becomes necessary now to inquire into the behavior and transmission of the genes which are associated with these nuclear structures. As we have seen, haploid organisms have one set of chromosomes and accordingly contain just one set of genes. The situation is quite different in diploid organisms where each chromosome, and thus each gene, normally is paired. Each gene located at a certain site in one partner of the chromosome pair is matched by a corresponding gene at a similar site in the homologous chromosome. Quite frequently the members of such gene pairs may differ from each other because not all the genes contributed by one parent are necessarily identical with those contributed by the other parent. Two such genes located at corresponding sites, or *loci*, of homologous chromosomes but producing somewhat different effects are called *alleles*. When a gene derived from one parent differs from the corresponding gene contributed by the other parent, the effect of only one of the two genes may dominate, covering up the detectable effects of the other (Fig. 6). Such genes are called *dominant* in contrast to *recessive* genes

which must be present in duplicate in order to manifest their activity. For example, in his pioneering studies, Gregor Mendel crossed a pure-breeding red-flowered pea plant with a pure-breeding white-flowered one and observed that the progeny showed red flowers only. He, therefore, described red flower color as a dominant character and white color as a recessive character. Nowadays this would be expressed more accurately by stating that the effect of the gene determining the development of red color is dominant, whereas the effect of the gene controlling white color is recessive. Mendel clearly recognized that even though the progeny of the

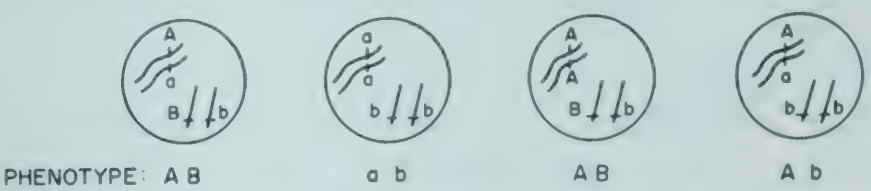


Fig. 6. Diagram showing the genotype and phenotype of cells carrying various combinations of dominant genes (A and B) and recessive genes (a and b).

above cross failed to show white flowers the gene for whiteness was not lost. When he permitted self-fertilization between the red hybrids he found that the white color flower reappeared in a certain proportion of members of the next generation. By assigning the symbol R to the dominant gene controlling red flower color, and the symbol r to the recessive gene controlling white flower color,[2] the results can be summarized in the following scheme:

Parents (P_1):	RR (red)		x		rr (white)			
P_1 gametes:	R				r			
First generation (F_1):			Rr			x	Rr	
F_1 gametes:		R		r		R		r
Second generation (F_2):		RR (red)		Rr (red)		Rr (red)		rr (white)

It has been recognized that dominance is not an all or none

[2] It has become customary to write the symbols for dominant genes in capital letters, those for recessive genes in lower case letters. Different alleles are frequently designated by superscripts, the allele conferring physiological activity being designated by a "+" sign. For example, Lac^+ designates a gene controlling lactose fermentation (or a mutant capable of fermenting lactose); Lac^- designates the absence of this ability.

property but rather is quantitative. All different degrees of dominance have been observed and it has been established that the dominant effects of a given gene can be affected both by environmental agents and by the activity of certain other genes.

Linkage and segregation

Each chromosome contains a large number of genes, and since chromosomes are usually transmitted as complete units, all the

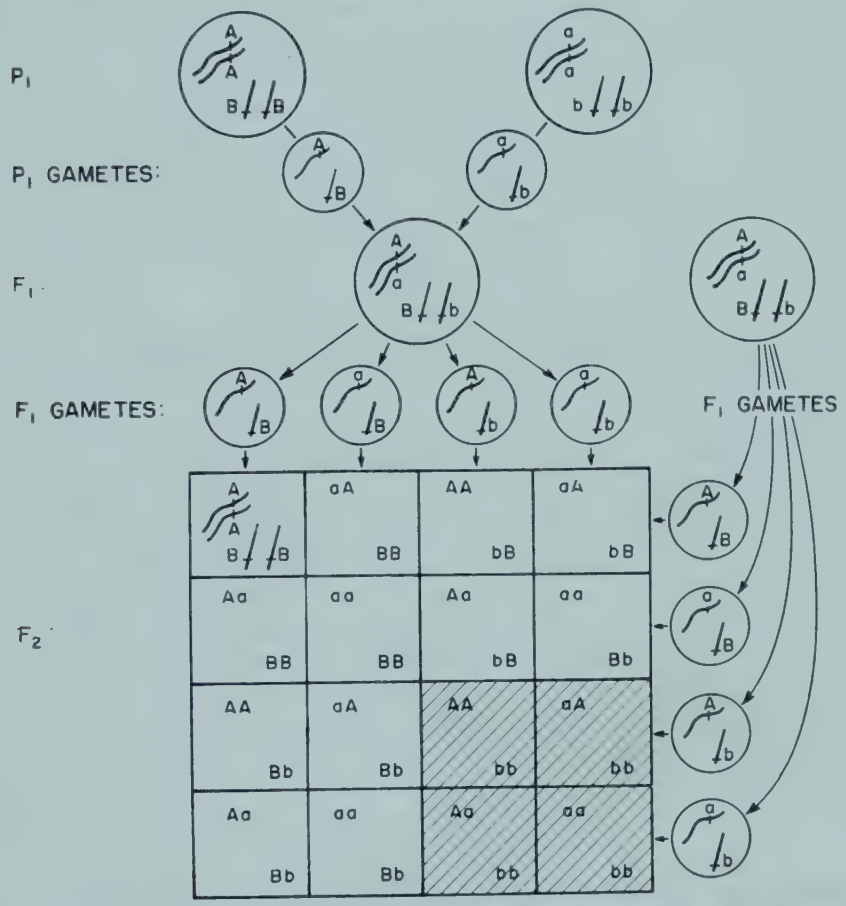


Fig. 7. Diagram showing the segregation, during meiosis, of genes associated with homologous chromosomes as well as the independent assortment of genes associated with non-homologous chromosomes. The results of random union among the four types of gamete formed by the F_1 heterozygote are shown in the F_2 checkerboard. The shaded areas in the checkerboard indicate that only one fourth of the F_2 will be homozygous for the recessive gene b and thus will manifest its phenotypic effects.

genes within one chromosome are similarly transmitted as a unit to the progeny. The geneticists call such genes *linked genes* and talk of linkage groups, corresponding to chromosomes, and linked

inheritance. Occasionally such linked genes separate in the course of meiosis by a process known as "*crossing-over.*" This phenomenon will be considered further in Chapter 11. In contrast, genes located in different, non-homologous, chromosomes may be distributed at random with the corresponding chromosomes during the reduction division that occurs during the maturation of sex cells (see Fig. 4). Such genes may separate during the development of individual sex cells; following fertilization the progeny will show a corresponding segregation of traits in individuals (Fig. 7). Gregor Mendel first studied such segregations experimentally and observed that they occurred in certain consistent proportions. The segregation ratios occurring among diploid, sexually reproducing, individuals are known as one of the Mendelian principles. Since they play a very minor role in bacterial genetics so far, we shall not discuss them further. The interested reader is referred to biology or genetics texts for more detailed information.

THE STABILITY OF HEREDITARY DETERMINANTS

Mutation

Reference has already been made to the differences which may exist in individual genes, and the corresponding hereditary traits, contributed by either parent in higher organisms. A widely held concept attributes the origin of such changes to relatively stable rearrangements occurring within the macromolecule we call a gene. Genes, therefore, are not absolutely stable but are apt to change or *mutate*, a process referred to as *mutation*. Such changes occur spontaneously and independently of the environment at a rather constant frequency for individual genes. In higher organisms mutations have been found to occur at a rate of approximately 1×10^{-5} to 1×10^{-8} per gene per generation. Such a change normally involves only one member of the duplicate set of genes present in a diploid organism. A mutated gene, therefore, will initially coexist in a cell containing a corresponding non-mutated gene (normal allele). If the mutant gene is recessive, it will not be able to express its effect in the presence of its normal allele and will only exert its influence in later generations if it is matched within the same cell by a corresponding mutant gene. Accordingly, in higher organisms where diploidy is the rule, a recessive mutation can never express itself until several generations after its occurrence. In haploid organisms, as bacteria are usually presumed to

be, a mutant recessive gene will not be suppressed by a normal allele and thus mutations theoretically can express themselves shortly after their occurrence (see p. 75).

At this point it must be mentioned that even in haploid organisms with one set of genes in each nucleus a condition may occur which suppresses the expression of recessive genes and the immedi-

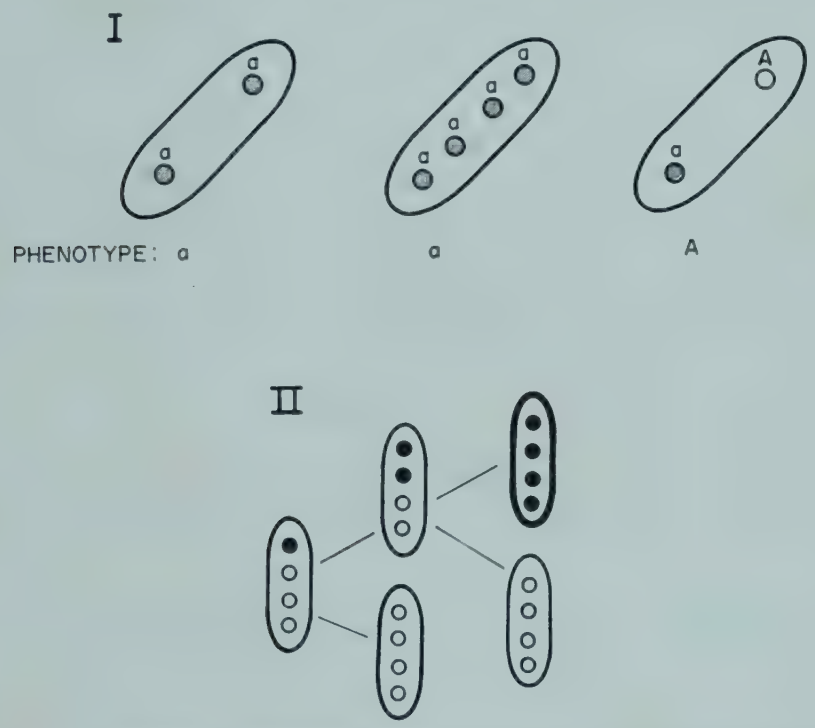


Fig. 8. *I*, Diagram of multinucleate cells. Nuclei with identical genotype are indicated by equal shading. Cell at right is heterocaryotic. *II*, Diagram illustrating the delay in phenotypic expression of a recessive mutation that occurs in only one of the nuclei (black nucleus in above diagram) of a multinucleate cell.

ate phenotypic manifestation of a recessive mutation. Such a condition, frequently encountered among fungi and apparently of considerable importance in bacteria as well, occurs when nuclear divisions proceed more rapidly than cell divisions. This leads to the existence of several nuclei within a single cell (multinucleate cell) and thereby causes the coexistence of duplicate or multiple sets of genes just as in the case of diploid or polyploid cells. If cells contain several nuclei that differ from each other in their respective complements of genes they are called *heterocaryotic* and the condition is described as *heterocaryosis* (Fig. 8). A recessive gene exist-

ing in only one of the nuclei of a heterocaryotic cell will fail to express its effect just as a recessive gene in the presence of its normal allele will fail to manifest its influence in a uninuclear diploid cell. Yet a subsequent reduction of the multinucleate state to the uninucleate condition will enable the recessive gene to exert its influence. Dominant genes, of course, are capable of exerting their influence in heterocaryotic as well as diploid cells. Most mutations, however, are recessive in nature.

Mutagens

The normal or spontaneous rate of mutation of individual genes can be significantly increased by certain environmental agents, such as x-rays, ultraviolet irradiation and nitrogen mustard (see p. 69). Agents that are capable of enhancing the normal rate of mutation are called mutagens and the resulting changes are referred to as *induced mutations*.

Chromosomal changes

Studies with higher organisms have shown that spontaneous as well as induced genetic changes can be caused in several ways.

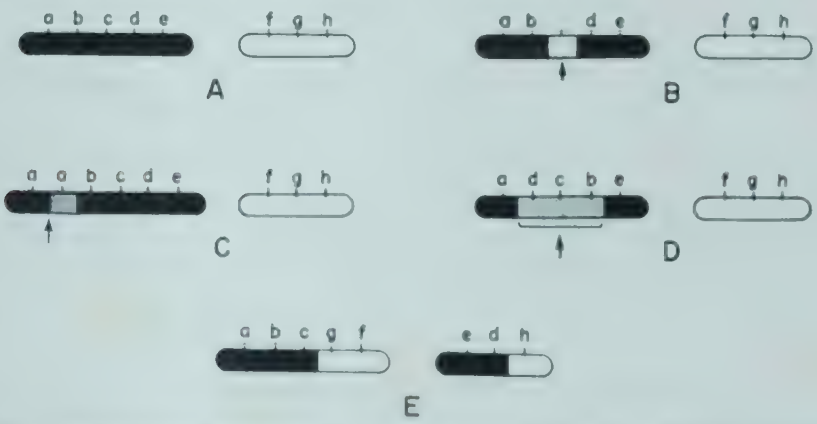


Fig. 9. Diagrammatic representation of several types of chromosomal aberrations. Each sketch shows two non-homologous chromosomes. *A*, Normal chromosomes; *B*, deletion; *C*, duplication; *D*, inversion; *E*, translocation.

Reference has already been made to molecular changes presumed to occur within specific, minute areas of the chromosomes, i.e., within the genes proper. Relatively permanent changes in genic control and thus in specific hereditary characteristics may also occur due to larger structural changes within or between chromosomes.

such as *deletions* of detectable regions of the chromosome, *duplications* of specific chromosome regions, *inversions* within an individual chromosome or *translocations* of portions of one chromosome to another non-homologous chromosome (see Fig. 9). Considerable significance has been attached in recent years to the fact that when minute portions of a chromosome, i.e., genes, are transferred, either spontaneously or following exposure to a mutagen, to a different region of the same chromosome, or are transferred to another chromosome, the activity of dislocated genes may be altered. This so-called *position effect* and some other recent data have given rise to the belief that genes, after all, may not function as entirely independent units, physically and physiologically separated from each other, but may act in unison thus making the chromosome, rather than its subunit represented by the "classical gene," the basic functional unit.[3] In a somewhat crude manner this newer concept may be compared to the specificity of protein macromolecules, in which the amino acids represent specific subunits but where the protein specificity is controlled by the arrangement and type of amino acids making up the macromolecule. However, for the sake of simplicity we shall continue here to refer to the gene as if it were an independent physical and physiologically active unit, a concept which, though oversimplified, has certainly proven its usefulness as a working hypothesis in genetic research for the last forty years. Yet even those who have regarded genes as the basic physical and physiological unit of hereditary traits clearly recognized that there is a considerable degree of interaction between the effects produced by individual genes. We shall elaborate on this in the following section which will inquire into the mode of action of genes.

GENE ACTION

Genetic studies on higher organisms, plants as well as animals, have contributed a great deal of indirect information on the action of genes. Such information has been obtained primarily through a knowledge of the effects which a specific gene may produce in the presence or absence of other specific genes or under different environmental conditions. By necessity, most of these studies had to be restricted to observations on easily detectable phenotypic

[3] Goldschmidt, R., Cold Spring Harbor Symp. Quant. Biol., *16*:1, 1951; McClintock, B., Cold Spring Harbor Symp. Quant. Biol., *16*:13, 1951.

characteristics of the experimental animal or plant, i.e., the end products of gene action, and their correlation with a known genotype, the latter being subject to analysis by cross-breeding and cytological procedures. In such analyses a wide gap persisted between the evocator of the reactions, the gene, and the final product, the detectable morphological or physiological characteristic. This gap involves a multitude of intermediate steps, represented in the case of higher organisms not only by the many reactions and interactions possible within individual cells, but also by the interactions between different cells.

In order to visualize the enormous complexity attendant upon such studies it is only necessary to think of an organism, or even of its subunits, the individual cells, as a highly complex machine where the genotype represents the master control board, each gene being somewhat akin to a specific switch regulating the movement of a specific gear. It is obvious that we cannot construct a blueprint of such a machine if we know only the manufactured product and the control board. However, we can make certain deductions about the minimum degree of complexity of the machine if we find that pulling a certain switch will consistently result in a specific alteration of the product. We would also learn that a high degree of integration between the gears and processes is necessary in order to get an acceptable product. We would further learn that one switch can directly or indirectly control many gears in this machine and that altering the speed of one gear may alter the speed of many subordinate gears connected with it. Furthermore, a specific action of this machine might not be dependent on pulling only one switch but could be controlled by pulling several switches at once.

This is exactly the type of information which has been obtained in studies with higher organisms. It was found that one gene may affect several not obviously related characteristics (*pleiotropic* genes with manifold or multiple effects); it is possible that the cause for such manifold effects may be one single effect upon a common precursor or pathway in the chain of reactions leading to the various affected characteristics. It was also found that several independent genes may affect one characteristic (*multiple factor* hypothesis). This is especially true of genes affecting quantitative characteristics such as size, shape and degree of coloration. Some genes have little or no effect by themselves but are able to modify

the effect of other specific genes significantly. Such genes are called *modifiers*. A particularly interesting group of modifiers is represented by the *suppressor* genes; these are genes capable of entirely suppressing the effect of other specific genes. Thus certain reversions in phenotype from a mutant characteristic to that of the original "wild" appearance may not always involve a reverse mutation of the specific gene controlling the particular characteristic. A mutant gene (A) may still be present but an independent mutation involving the activity of an entirely different gene (B), a suppressor gene, may have occurred which now suppresses the effects of the still unchanged gene A (see p. 96). Also, just as an arbitrary shift in a critical gear of a machine may break down the entire mechanism, many mutations are *lethal mutations,* interrupting or upsetting the normally harmonious integration of gene-controlled processes in such a manner that a viable organism or cell fails to result.

In some instances it has been possible in higher organisms to trace back gene-controlled processes several steps prior to the final expression of the hereditary characteristics. For example, certain processes controlled by genes affecting the development of an insect's wing have been traced back to the larval stage. Such studies reveal that genes control not only specific steps in a long chain of reactions but also the time at which reactions take place.[4] Furthermore, it has been demonstrated that many gene-controlled processes proceed in divergent pathways from a common precursor reaction (Fig. 10). This type of dependence of several end reactions upon a common root reaction illustrates in a highly simplified manner how genes may affect one or several reactions depending upon the level at which a specific gene may interfere with this chain of gene-initiated reactions. Thus a gene affecting level A in Figure 10 will affect all subsequent reactions, a gene interfering at level B will affect a lesser number of subsequent reactions, etc. From all this it may be seen that the final phenotype of an organism or a cell is the end result of a multitude of complex interactions between gene-controlled processes, which in turn can be modified temporarily by environmental conditions.

While studies on higher organisms revealed a great deal about the mechanics of gene-action, significant progress in understanding gene action in more meaningful biochemical terms was made pos-

[4] Goldschmidt, R., *Physiological Genetics,* McGraw-Hill, New York, 1937.

sible when microorganisms became the tools of genetic studies. In particular, the pioneering studies of Beadle and Tatum on the mold *Neurospora* contributed significantly to the more recent development of our concepts of gene action[5]. Such studies with fungi and, as we shall discuss later, with bacteria as well permitted an analysis of gene-controlled processes on a cellular level. The results of these studies indicate that genes are the basic determinants of the synthetic chemical activities of the cell and that they are capable of controlling specific enzymatic processes. In principle, this information was obtained by studying the effects of mutations affecting the cells'

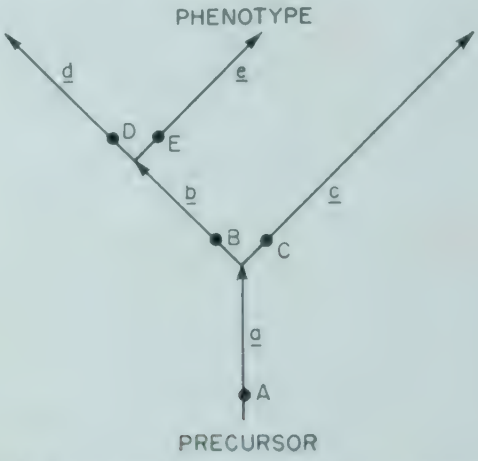


Fig. 10. Diagram of divergent pathways in one series of gene-controlled reactions.

ability to synthesize some of their growth-requirements. The wild-type *Neurospora* is able to synthesize all of its cellular constituents except biotin from carbohydrate and inorganic salts. Mutants were obtained which are incapable of performing certain biosynthetic steps and can grow only if a specific growth factor is supplied in the nutrient medium. Analysis of such "biochemical mutants" has revealed that specific genes are capable of controlling specific steps in a biosynthetic pathway. Thus compound E, required for the cells' function and growth, may be synthesized normally from precursors through steps A→B→C→D→E. Changes in gene "a" can interrupt step A→B and this interference can be repaired only by supplying the cell with preformed B, C, D, or E. Changes in another gene, "b," may interrupt step B→C, and this block can be overcome only if C, D, or E is supplied to the cell from external sources, etc. Such results demonstrate that specific genes

[5] Beadle, G. W., Chem. Rev., *37*:15, 1945.

control specific steps in a sequence of reactions, presumably by controlling the activity of the specific enzyme necessary to catalyze the synthetic reactions involved in each step. The finding that a change in one gene is capable of abolishing a single reaction in the synthesis of an essential metabolite gave rise to the "one gene—one enzyme hypothesis," a fruitful working-hypothesis which assumes that each gene is the precursor of a specific enzyme. However, recent studies indicate that this concept is probably oversimplified. Cases have been uncovered where changes in one gene appear to affect more than one reaction, and it has also been shown that in some mutants where a specific enzymatic reaction is lacking, the enzyme, though inactive, can actually be recovered from the cells.

Some investigators have even suggested that genes and enzymes are identical, and some support for such an assumption has come from calculations which indicate that there is sufficient space within a cell to accommodate all potentially active enzymes if they are present as one or a few enzyme molecules per cell[6]. Later experimental studies with bacteria, however, indicate that the observed rates of certain metabolic reactions cannot be accounted for on the basis of one enzyme molecule per cell. It has also been proposed that genes, rather than acting as precursors of enzymes, may normally act as inhibitors of enzymatic processes, inhibiting specific enzymatic reaction qualitatively as well as quantitatively[7]. This concept envisions that the extra-chromosomal material has the potential for many more enzyme reactions than are actually realized during the development and life of an organism or a cell; many of these potential enzyme reactions normally would be blocked by the chromosomal constituents which would act somewhat like a buffer system. So far, none of the discussed interpretations can fully account for all recorded observations and the problem of the primary stages of gene action, including the manner in which the gene products are transferred from the nucleus to the cytoplasm, remains largely a matter of speculation. However, it can be stated with assurance that genes play a decisive role in controlling the intermediate steps and the timing of biosynthetic processes which in the final analysis must form the basis for physiologic activities as well as for the development of morphologic characteristics.

[6] McIlwain, H., Nature, *158:*898, 1946.

[7] Braun, W., Science, *104:*38, 1946.

It is important to note that analyses of several specific gene-controlled biosynthetic pathways in different organisms, e.g., *Drosophila*, *Neurospora*, and bacteria, have revealed the occurrence of identical sequences of biosynthetic reactions. This demonstrates that there is a close relationship between all living things both in the evolutionary sense and from the standpoint of the generality of biosynthetic and genetic processes.

EXTRA-NUCLEAR DETERMINANTS OF HEREDITARY CHARACTERISTICS

So far we have discussed only the nucleus and its chromosomes as the site of genetic determinants. In doing so we have followed the historical development of genetic information because until quite recently there was a tendency to regard the nuclear components as the sole site of the determinants of hereditary characteristics. Although there is no doubt that the nuclear constituents play the major role in controlling hereditary traits, recent studies, particularly with such microorganisms as *Paramecium* and yeast, have revealed interesting facts about the role of cytoplasmic factors which, in conjunction with nuclear factors, can control certain hereditary traits (*cytoplasmic inheritance*)[8]. The existence of similar extra-nuclear factors in bacteria is still undetermined. Nevertheless, a brief description of the facts known in other organisms will be of value because comparable phenomena may be revealed in bacteria once the required techniques become available.

Prerequisite to an analysis of cytoplasmic inheritance is sexual propagation, which permits the detection of nuclear control through the phenomenon of segregation; cytoplasmic factors that control hereditary traits will be transmitted without segregation through succeeding generations even after matings with a parent that does not carry the particular trait. Since in higher organisms the bulk of the cytoplasm is contributed by the female gamete and only a negligible portion by the male gamete, cytoplasmic factors are transmitted through the females in plants and animals.

Cytoplasmic inheritance in plants and Drosophila

It has been known for many years that in a wide variety of plants certain self-reproducing cytoplasmic particles, plastids, are apt to mutate and thereby cause leaf variegation, in which the normal

[8] Caspari, E., Adv. in Genetics, *2*:1, 1948.

green tissue is spotted with irregular patches of lesser coloration. This characteristic is maternally inherited, and it has been shown that plastid mutation is dependent on the presence of specific nuclear genes. Other cases of cytoplasmic inheritance in plants, in which plastids are not involved, also have been found. An interesting case of extra-chromosomal inheritance in animals has been uncovered recently by l'Heritier; it involves sensitivity of *Drosophila* to carbon dioxide which was found to be transmitted by self-perpetuating cytoplasmic components that in practically all respects behave like a virus.

The killer characteristic of Paramecium

The most interesting information on cytoplasmic inheritance has been obtained by Sonneborn and co-workers[9] in their studies with the infusorian *Paramecium aurelia*. Their earlier studies dealt with the inheritance of the "killer" characteristic which causes certain strains to secrete a substance, called paramecin, that kills "sensitive" strains of the same species. Paramecia can reproduce in two ways: (1) by asexual fission giving rise to *clones*, i.e., the progeny of a single cell, containing genotypically identical animals, or (2) by sexual conjugation which leads to the fusion of two nuclei, each originating from one of the mates (in some instances both nuclei can originate from one animal only, a process termed *"autogamy"*). It was found that during asexual reproduction killer individuals always give rise to killer clones, and sensitive individuals produce sensitive animals. By analyzing the progeny of matings between killer individuals and sensitive animals it was determined that killers possess a dominant nuclear gene K, whereas sensitive individuals usually carry the recessive allele *k*, actually two *k* genes (*kk*) since Paramecia are diploid. According to the usual behavior of such gene-controlled traits it would be expected that all K*k* and KK animals would be killers (remember K is dominant over *k*), whereas all *kk* animals would be sensitives. However, it was found that matings of KK with *kk* could result in K*k* animals which gave rise to killer and sensitive clones, even though all the resulting individuals carried the K*k* gene combination (Fig. 11 A). This result was obtained only if the period of conjugation between the two

[9] See Sonneborn, T. M., Proc. Nat. Acad. Sci., *34*:413, 1948; *In* Centennial, publ. by AAAS: 243, 1949; Heredity, *4*:11, 1950; *In* Science in Progress, 7th Series, Yale Univ. Press, New Haven, 1951.

parents was brief. After a prolonged period of conjugation which permitted not only the exchange of nuclei but also an exchange of cytoplasm between the mated individuals, animals with killer characteristics resulted (Fig. 11 B). These apparently odd facts were explained by the presence of cytoplasmic particles, termed *kappa*, which are necessary to produce the killer characteristics. *Kappa*

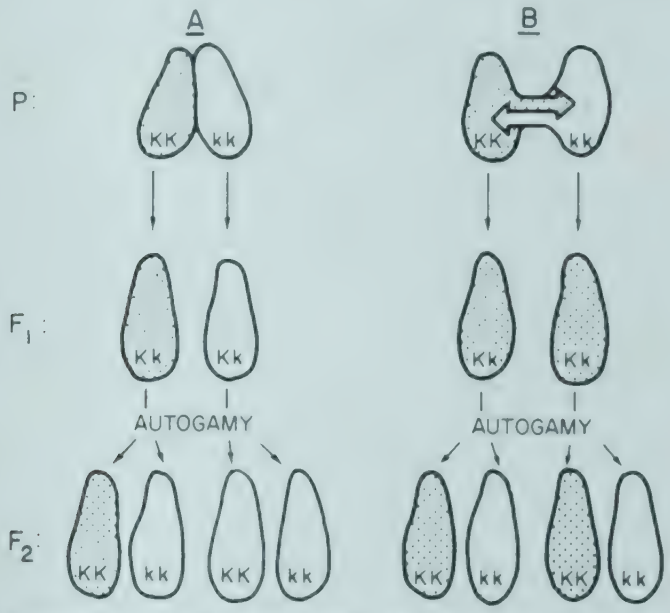


Fig. 11. Diagram of the inheritance of the killer and sensitive traits in *Paramecium aurelia*. Stippling symbolizes killers (and presence of *kappa*), white symbolizes sensitives. The nuclear condition is indicated by the symbols within animals. *P*, Parents; F_1, first generation, obtained by sexual conjugation; F_2, second generation, obtained by self-fertilization in animals of first generation. Column *A* shows results when no cytoplasm is exchanged between parents; column *B* when the parents exchange cytoplasm. (After Sonneborn.)

can only be maintained in the presence of the gene K and mating between KK animals, containing *kappa*, and *kk* animals, without *kappa*, thus resulted in two types of animals if the period of conjugation was brief: K*k* with *kappa* yielding a killer, and K*k* without *kappa* yielding a sensitive animal despite the presence of the K gene. Following a conjugation that lasts long enough to permit an exchange of cytoplasm between the parents the K*k* progeny will consist of killers only since *kappa* particles are now present in all animals. The cytoplasmic *kappa* particles represent autocatalytic (self-reproducing) particles and it has been possible to identify and count them under the microscope; they give a staining reaction that

is positive for desoxyribonucleic acid. The rate of multiplication of *kappa* can differ from that of the nucleus. Thus, under certain environmental conditions it has been possible to cause a loss of *kappa* in KK clones by permitting nuclear and cellular fission to occur at such a fast rate that the propagation of *kappa* cannot keep up and *kappa* is lost. Under these conditions killers are transformed into sensitives. This well-analyzed phenomenon of the killer trait provides an excellent example of the interactions that are possible between nuclear genes and autocatalytic cytoplasmic factors. It demonstrates that hereditary traits can be controlled by cytoplasmic elements which, in turn, are dependent upon nuclear factors for their maintenance. Even if *kappa*, as some have pointed out, meets in almost every respect the criteria for an intracellular parasite, it remains remarkable that this symbiosis is gene-controlled.

Antigenic transformation in Paramecium

Another remarkable case of cytoplasmic inheritance described by Sonneborn's group in *Paramecium* concerns the control of antigenic characteristics. Antigenically distinct types are known which, when maintained under standard environmental conditions, infrequently but spontaneously may give rise to new antigenic types. Breeding experiments have shown that the different antigenic types are identical in their genes and differ only in their cytoplasm. However, in contrast to the *kappa* phenomenon, these cytoplasmic differences cannot be associated with microscopically detectable cytoplasmic particles. Under the influence of certain environmental agents it has been possible to transform these antigenic characteristics. For example, exposure of type D to anti-D serum will result in the transformation of practically all the exposed animals or their progeny to different antigenic types, e.g., types A, B, C, E or G. The transformed animals subsequently reproduce true to the new type, even through sexual reproduction, but the transformations are reversible if transformed animals are again exposed to a specific antiserum. For example, D animals $\xrightarrow[\text{serum}]{\text{anti-D}}$ A clones $\xrightarrow[\text{serum}]{\text{anti-A}}$ D clones. Similar antigenic transformations can also be accomplished by cultivating animals under different nutritive conditions or at extreme temperatures. For example, type D stays D at 26° C, but transforms after a few fissions to type A at 32° C, to type B at 12° C. Following return to 26° C such transformed animals con-

tinue to reproduce true to the new type. When similar studies were carried out with another stock of *Paramecium*, it became apparent that even though the general mechanism of transformation was similar, some of the resulting antigenic types were different from those obtained in extensive studies with the first stock. This strain specificity indicated a controlling influence of the genotype. Appropriate cross-breeding tests confirmed that the range of potential antigenic transformations was gene-controlled, whereas the cytoplasm determined which of the several potential antigens was actually formed. As Sonneborn has expressed it, in describing the production of antigen F, "the presence of a certain gene merely determines the *capacity* to form substance F, it does not *assure* formation of F, for the cytoplasm controls which one of the alternative substances will be formed." The manner in which this cytoplasmic control is produced is still not entirely clear. One possible interpretation suggests that autocatalytic precursors of the potentially active antigens are present in different proportions in the cytoplasm, and that only those specific entities present in the majority develop into active antigens. Certain environmental conditions may cause a shift in the relative proportion of these antigen-precursors, thus producing a predominance of the precursors of a different antigen and their subsequent development into an active, detectable antigen. Shifts in intracellular metabolite levels, under the influence of the environmental agents causing antigenic transformation, may be responsible for the shift in the intracellular population of such competing cytoplasmic elements[10]. However, it is not absolutely necessary to invoke the participation of self-duplicating cytoplasmic particles in order to explain the recorded phenomena. For example, Delbrück has suggested competitive processes among cytoplasmic reactions that may involve inhibition of some steps in the synthesis of all but one antigen-determining substance in the presence of high concentrations of one of the specific substances (hypothesis of mutually inhibitory alternative steady states). More recent findings by Beale[11] have opened the way to still another interpretation. Beale has observed that at least in one variety of *Paramecium*, genes at different loci are concerned with antigen production. The state of the cytoplasm, modifiable by environmental changes, appears to control selectively which of

[10] Braun, W., Amer. Naturalist, *86*:355, 1952.
[11] Beale, G. H., Genetics, *37*:62, 1952.

these genes can express itself. The nature of the factors concerned with this establishment of a particular cytoplasmic state remains unknown.

It should be stressed at this point that there is no evidence that changes in antigenic characteristics of bacteria involve control through cytoplasmic factors similar to that uncovered in antigenic transformation of *Paramecium*. The antigenic changes normally encountered in bacterial populations involve the gradual establishment of one or a few antigenically different, spontaneous mutants (p. 127) rather than a transformation of the characteristics of a majority of cells previously exposed to specific environmental conditions as has been found with *Paramecium*.

Cytoplasmic inheritance in yeast

Ephrussi and co-workers[12] discovered still another example of cytoplasmic inheritance in their studies with a "small colony" mutant of yeast. They demonstrated that the small colony characteristic was associated with an impairment of the cytochrome oxidase system. Breeding experiments revealed that in this case the respiratory enzymes are controlled by cytoplasmic factors, presumably associated with mitochondria; they also showed that the maintenance of these cytoplasmic factors, as in the case of *kappa*, is under the control of nuclear genes. A specific environmental condition, exposure to acriflavine, was found to inhibit the autocatalytic propagation of the cytoplasmic factors controlling the cytochrome system, and such exposures resulted in a rapid transformation of the progeny of normal cells into relatively stable small colony types.

Interactions between nuclear and cytoplasmic elements

The above cited cases of cytoplasmic inheritance are regarded at present as exceptional cases rather than widespread phenomena. The fact that cytoplasmic control of hereditary characteristics has been observed only in a few specific instances, however, may be due in part to a lack of appropriate techniques for its detection in many other organisms, including bacteria. In a few, apparently exceptional, cases of bacterial variation where changes in hereditary characteristics occur at unusually high rates following exposure to

[12] Ephrussi, B. and Hottinguer, H., Cold Spring Harbor Symp. Quant. Biol., *16*:75, 1951.

specific environmental conditions, and are subsequently maintained under different environmental conditions, the involvement of cytoplasmic factors may be suspected. Some of these suspicious examples will be pointed out later (pp. 101, 103). But even where the involvement of autocatalytic cytoplasmic elements in the determination of hereditary characteristics has been recognized, as in the cases described above, it has been found that the cytoplasmic entities are not entirely autonomous and that their appearance or maintenance depends on the presence of specific nuclear genes. Thus, even in the case of cytoplasmic inheritance, the genes remain the major determinants, and the problem becomes merely one of interactions between nuclear and cytoplasmic elements.

ENZYMATIC ADAPTATION

The studies on cytoplasmic inheritance have shown that genes are able to control the potential activity of autocatalytic cytoplasmic entities. A similar mechanism of gene control was revealed in investigations that were concerned with a special case of environmental modification known as *enzymatic adaptation* or *adaptive enzyme formation*. This phenomenon, detected in molds and yeasts, as well as bacteria, involves the ability of cells to activate specific enzymes under the influence of a specific substrate. In contrast to the cytoplasmic factors like *kappa*, the *Paramecium* antigens, and the cytochrome activity, the adaptive enzymes once brought into play in the presence of a specific substrate usually will not maintain their activity for prolonged periods following transfer to a different environment lacking the particular substrate. Since much of the information on adaptive enzyme formation was obtained in studies with yeast, we shall review some of its basic manifestations here and refer in a later section (Chapter 10) to specific data obtained with bacteria.

A typical example of enzymatic adaptation is the failure of certain yeast strains to produce galactozymase when grown in the absence of galactose. However, when galactose is added to the medium the cells will produce the enzyme after a brief lag and are then able to ferment this sugar. Similarly, cells pregrown in the presence of maltose are capable of fermenting this sugar, whereas identical cells grown in the absence of this substrate fail to produce maltozymase. Once the cells have acquired this ability to ferment a certain sugar they lose it subsequently, usually after 6 to 7

generations, if maintained in the absence of the substrate. Breeding experiments demonstrated that the potential ability to form certain enzymes is gene-controlled. Spiegelman[13], Lindegren[14], Monod[15], and Winge[16] are among the several investigators who have analyzed this phenomenon extensively. They have demonstrated that these particular population changes are not due to the mutation of a few cells and their subsequent selection, but are due to the capacity of genetically unaltered cells to form, or to activate under the influence of the specific substrate, a previously inactive enzyme. It is important to distinguish between this particular phenomenon which involves changes in all or most cells in a suitably exposed population and the more frequently observed population changes due to mutation and selection which initially involve changes in a few cells only. Selection obviously cannot be involved if enzymatic changes can take place in the absence of cell multiplication, and the most important proof supplied for the existence of true enzymatic adaptation is the fact that it can occur in resting cells. It is generally believed that this phenomenon involves the autocatalytic propagation of cytoplasmic entities under the influence of the specific substrate, and Spiegelman[13] has obtained results which indicate that it is the apoenzyme or protein moiety of the enzyme complex which is modified during the course of adaptation. Enzymatic adaptation appears to require an exogenous or endogenous energy source. Because of this energy requirement competitive interactions between enzyme-forming systems may result when cells are brought simultaneously into contact with two or more substrates to which they can adapt[15, 17] (see p. 181). Finally, calculations by Spiegelman[18] based upon experimental data with yeast indicate that "particulate (cytoplasmic) elements responsible for enzyme formation are being randomly distributed between mother and daughter cells." His data indicate that the initial number of these elements in an adapted cell is about 100 and that the minimal number required for

[13] Spiegelman, S., Chapter 6 in *The Enzymes*, Ed. J. B. Sumner and K. Myrbäck, Academic Press, Inc., New York, 1950.

[14] Lindegren, C. C., *The Yeast Cell, Its Genetics and Cytology*, Educational Publishers, St. Louis, 1949.

[15] Monod, J., Growth, *11*:223, 1947.

[16] Winge, O. and Roberts, C., Compt. rend. trav. lab. Carlsberg, Ser. physiol., *25*:36, 1950.

[17] Spiegelman, S. and Dunn, R., J. Gen. Physiol., *31*:153, 1947.

[18] Spiegelman, S., Cold Spring Harbor Symp. Quant. Biol., *16*:87, 1951.

the positive phenotype is in the neighborhood of 1. The actual nature of these cytoplasmic elements is as yet unknown.

POPULATION CHANGES

In genetic studies with higher organisms considerable information has been obtained on the fate of related organisms with different genotypes when they have to compete with each other in a population. These studies have established the general validity of the principles of natural selection and survival of the fittest pronounced a century ago by Darwin.

In both sexually and asexually reproducing organisms mutation causes genic heterogeneity in populations. In addition, considerable heterogeneity is produced in populations consisting of sexually reproducing organisms by the fact that parents usually contribute dissimilar genotypes to their offspring. Experiments have demonstrated that individuals with different genotypes have different chances of survival, or different *selective values*, under given environmental conditions. To choose a simple example, a wingless insect mutant will have a very poor chance of survival under most environmental conditions where it competes with its winged brothers and sisters; yet such a mutant will attain a high selective value on a small windy island, e.g. on an atoll, where the winged relatives are constantly in danger of being blown into the ocean. Similarly, a fungus mutant requiring a specific vitamin for growth and reproduction will fail to survive in competition with the "wild" type capable of synthesizing this growth factor, unless the specific requirement is present in its environment. However, when the growth factor is supplied, the mutant may well have a higher selective value than the wild type if, for instance, it has a higher reproductive rate. A mutation that is deleterious in one environment, therefore, may be of considerable value under another set of environmental conditions. The so-called normal or wild representatives of present species are merely the mutants which in the course of evolution had the best chances of becoming established under the prevailing conditions. The genic heterogeneity present in every natural population endows such populations with enormous plasticity of response towards changes in environmental conditions, permitting the survival of those members of the heterogeneous population that are best adapted to the existing environmental

conditions. The individual members of a population do not undergo stable changes in response to the environment, but the population can undergo changes because environmental conditions cause the selective survival of the fittest, genetically different, members of the population. Undirected, spontaneous mutants and their selection thus can contribute to gradual population changes, which may give the over-all appearance of having been directed.

It has frequently been pointed out that the majority of mutations that have been detected under laboratory conditions are unfavorable rather than beneficial. In fact, a large number of experimentally induced mutants have proved to be lethal. However, any generalization regarding the lack of selective value of experimental mutants is misleading. It should be remembered that species as they exist in nature today are the products of millions of years of selection for beneficial mutational steps. Therefore, the mutants obtained in the laboratory may include primarily those which have already been eliminated in the history of the species. As soon as organisms are faced with a new set of environmental conditions not previously encountered in the evolutionary history of the species, mutations that were previously of little or no value may become advantageous. Witness, for example, the isolation of streptomycin-resistant bacterial mutants which are definitely beneficial for the organisms under appropriate environmental conditions. Finally, it has been established with higher organisms that a mutant gene unfavorable in the presence of certain genes may produce a high selective value if it is present in combination with certain other genes[19].

CONCLUSIONS

The preceding information obtained in genetic studies with higher organisms has revealed some general principles which are of importance for problems of bacterial genetics and may be summarized as follows:

a. Hereditary characteristics, including the appearance, structure and physiology of an organism, are under the primary control of self-duplicating nuclear elements which in sexual as well as in asexual propagation are transmitted from generation to generation.

[19] For further information see: Dobzhansky, Th., *Genetics and the Origin of Species,* Columbia University Press, New York, 1951; Dodson, E. O., *Textbook of Evolution,* W. B. Saunders Co., Philadelphia, 1952.

b. While these nuclear elements, genes, determine the reaction range of an organism, the final expression (phenotype) of a given characteristic can be modified by existing environmental conditions.

c. Environmentally induced modifications of gene-controlled characteristics are maintained only as long as the specific modifying environmental conditions persist; acquired characteristics are not inherited. In a few exceptional instances, e.g., cytoplasmic inheritance, certain environmental conditions may cause the appearance of new heritable characteristics that are maintained even after a change of environment; yet even this apparently rare phenomenon is under gene-control since the range of such potential transformations is determined by specific genes.

d. Genes apparently act by controlling the rate and specificity of biosynthetic processes.

e. One gene may affect several characteristics, but several genes may also interact to affect one particular characteristic.

f. Genes are not absolutely stable and may occasionally change (mutate) spontaneously, causing a change in the characteristic(s) which they control. The rate of mutation is constant for a given gene but may differ between genes. Certain environmental agents are capable of increasing the spontaneous mutation rate.

g. Many mutational changes are associated with structural changes in the gene-carriers, the chromosomes.

h. Although genes are hypothetical units, there is a material basis for heredity. In the transmission of hereditary determinants a direct correlation exists between the distribution of the chromosomes, as evidenced by cytologic observations, and the distribution of the genes associated with the chromosomes, as demonstrated by breeding experiments

i. In sexually reproducing organisms certain laws exist for the mode of distribution of chromosomes and their genes among the progeny, producing the phenomena of segregation, linkage and recombination of genes and the characteristics which they control.

j. The chief sources of variation among the characteristics of related organisms are (1) genic differences due to mutation of individual genes, or due to new assortment of genes in the case of sexual reproduction, where parents of dissimilar genotype contribute different genes to the offspring, or (2) environmentally induced differences causing, as a rule, only temporary changes of the phenotype.

k. Within a population of genetically different members, the opportunity for survival and propagation under given environmental conditions is not the same for each individual. Different genotypes have different selective values. The influence of the environment, as a selective force, leads to the survival of the fittest; selection superimposed upon genic differences thus plays an important role in population changes and evolutionary processes.

CHAPTER 2

The Evolution of Bacterial Genetics

In the following chapters it will be seen how studies with bacteria reflect and extend many of the general principles of genetics uncovered in research with higher organisms. However, this recognition of the universal applicability of genetic principles to all living organisms, including bacteria and viruses, is a fairly recent development. With few exceptions geneticists failed to extend their interest into the field of bacteriology until the early 1940's, while bacteriologists, though cognizant of bacterial variability since the days of Pasteur, chose interpretations that more often than not were unrelated to established genetic principles. The ideas of direct adaptation and inheritance of acquired characteristics were predominant in the older bacteriological literature. Bacteriology thus won the dubious distinction of being known as the last stronghold of Lamarckism. (The French biologist Lamarck, 1744–1829, was an early and prominent proponent of the now discredited idea of inheritance of acquired characteristics and his theory of biological evolution was based on such ideas.) Yet this retention of Lamarckian ideas among bacteriologists is not too surprising if one remembers that in studies with bacteria one usually deals with very large populations of organisms that can propagate *asexually* at rapid rates. This can and did prevent the identification of changes in individual organisms. In practically all older studies, changes in heritable characteristics which may have occurred in single cells remained undetected until such altered cells had given rise to many progeny. What generally has been observed among bacteria, therefore, is a change in populations rather than a change in individual cells. Prior to the development of a recognized discipline of bacterial genetics, such population changes were interpreted primarily in four different ways: Many believed that they represented direct adaptation to environmental influences, involving changes caused by the environment in all or a few cells of a population (Fig. 12 A).

Others have proposed a major role of *Dauermodifikationen* (lasting modifications); these are environmentally induced adaptive changes which persist, without affecting any inherent factors, for many generations after the removal of the inductive environment (Fig. 12 C). Still others have interpreted bacterial population changes as orderly life-cycles in which the expression of each stage is dependent on the environmental conditions (Fig. 12 B). Only a minority group of early investigators recognized bacterial variation as a phenomenon that can involve the occurrence of spontaneous and undirected changes (mutation) in one or a few cells which are subsequently selected under appropriate environmental conditions (Fig. 12 D). The last interpretation, bringing the basic mechanism of changes among bacteria in line with those known in higher organisms, gained overwhelming acceptance when techniques were devised which permitted (1) a distinction between changes affecting either single cells or all cells within a bacterial population; (2) the differentiation between absence or presence of changes induced by the environment; and (3) the demonstration of increased rates of population changes following exposure of bacteria to known mutagenic agents. The early 1940's saw the rapid development and application of such techniques. The beginnings of an organized research field of bacterial genetics may be traced back to those years[1]. Nevertheless, the older literature still contains a large amount of valuable experimental material on the manifestations of bacterial variation, and most of these older data can now be aligned successfully with the newer interpretations regarding the underlying mechanisms of bacterial variation[2].

Following are references to some representative early investigators of bacterial variation, classified according to the concepts which they advanced in the interpretation of their results.

Adaptation

The views of Seppilli[3] and Manwaring[4] are representative of those who endowed bacteria with a hypothetical inherent potential of sufficient flexibility to permit direct adaptation to a multitude of environmental conditions. These workers entirely rejected the ap-

[1] See Luria, S. E., Bact. Rev., *11*:1, 1947.
[2] See Braun, W., Bact. Rev., *11*:75, 1947.
[3] Seppilli, A., 3rd Intern. Congr. Microb., New York, Rept. Proc., 162, 1939.
[4] Manwaring, W. H., Science, *79*:466, 1934.

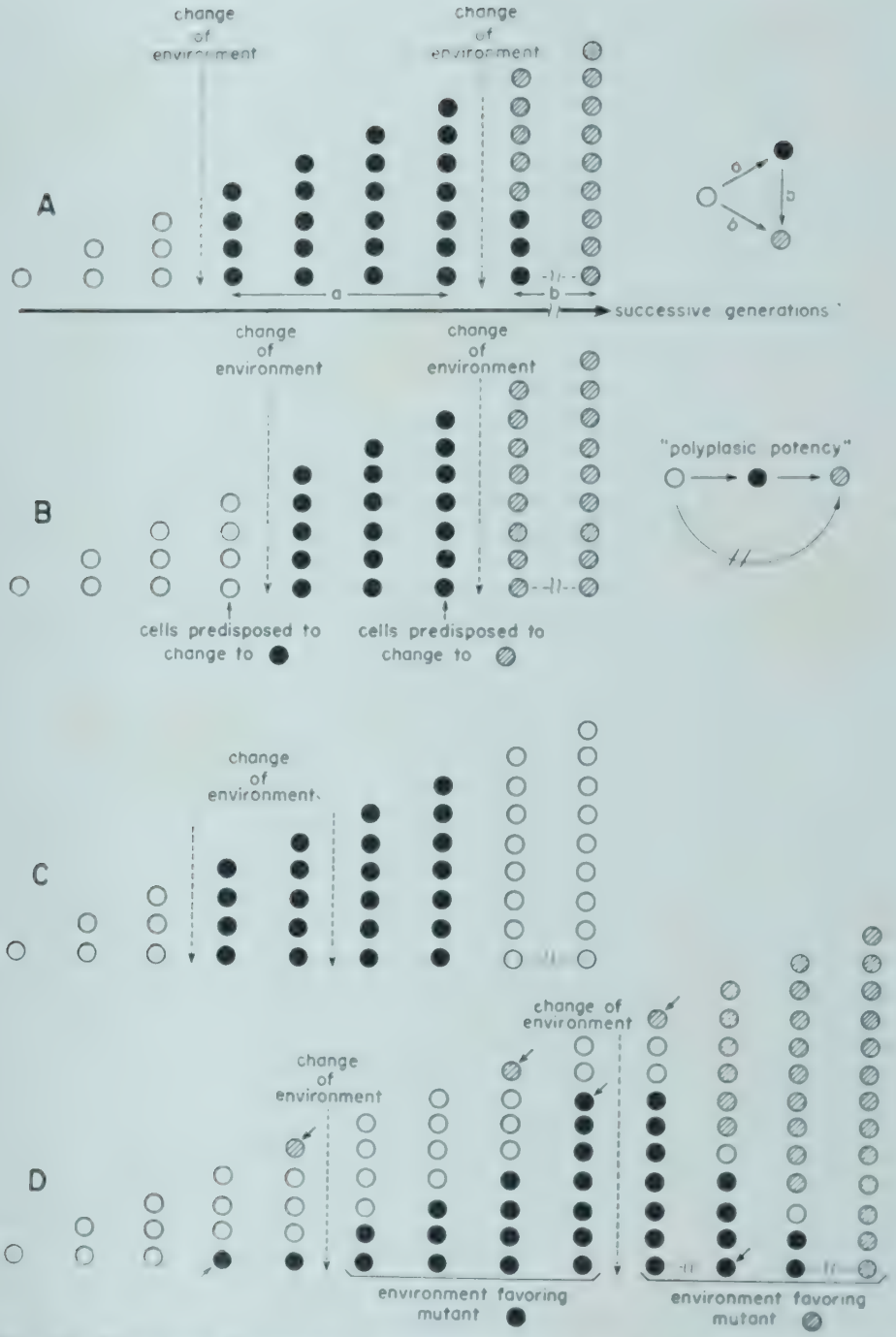


Fig. 12. Diagrams illustrating various concepts that have been proposed to explain the mechanism of variation within bacterial populations. *A. Adaptation*, involving a directed change of bacteria under the influence of the environment (environmental conditions indicated as *a* and *b*); *B. life-cycle*, involving an orderly succession of "culture phases," the expression of each stage depending on the environmental conditions; *C. Dauermodifikationen*, involving direct adaptations that will persist for prolonged periods following the removal of the environmental condition that induced the change; *D.*

plicability of the mutation concept to bacteria. In the absence of any critical evidence to the contrary, many bacteriologists used to share such views. Terms such as "adapted" and "acquired" became common in the numerous descriptive studies on bacterial variation which followed the Koch-Cohn era (1881) of extreme monomorphism. Even in the more recent literature one can still find a few proponents of the idea that direct adaptations may play a *major,* though not exclusive, role in bacterial variation[5].

Life-cycles

A concept that enjoyed considerable popularity, especially in the 1930's, was the "cyclogenic" or ontogenic theory advanced by Enderlein[6], Mellon[7], Hadley[8] and others. This concept attributed to bacteria the ability to go through orderly life-cycles, similar to the well-known cyclic stages of parasitic protozoa. The aforementioned workers were primarily concerned with studies on pathogenic bacteria and with changes involving antigenic, morphologic and virulence characteristics. To these variational phenomena they applied the term *dissociation,* first used by de Kruif[9] to denote the appearance, in a pure culture, of relatively stable but reversible types which differ usually in several characteristics from those of the parent types. The origin of the term "dissociation," as well as of ontogenic concepts, can actually be traced back to the bacteriological work of the 1870's, when the apparent fragmentation (i.e., "dissociation") of long filamentous cell structures into short bodies, and subsequent reversal of this change under proper conditions, was first observed. The later investigators extended the idea of dissociation and its ontogenic interpretation beyond this simple cellular change and applied it to the majority of variational phenomena observed in bacteria. In general, the cyclogenic or

[5] For example, Hinshelwood, C. N., *The Chemical Kinetics of the Bacterial Cell,* Oxford University Press, London, 1947.

[6] Enderlein, G., *Bakterien-Cyclogenie,* W. de Gruyter & Co., Berlin, 1925.

[7] Mellon, R. R., J. Bact., *11:*203, 1926; J. Bact. *44:*1, 1942.

[8] Hadley, P., J. Inf. Dis., *40:*1, 1927; J. Inf. Dis., *60:*129, 1937.

[9] De Kruif, P. H., J. Exp. Med. *33:*773, 1921.

mutation and selection, involving the spontaneous occurrence, independent of existing environmental conditions, of a few genetically different cells and their subsequent selection under appropriate environmental conditions. Arrows designate new mutants. The increase in population size is indicated schematically only by an increasing number of circles.

ontogenic theory stressed the polyphasic potencies of the bacterial cell and maintained that the "chief culture phases" (e.g., antigenically smooth, mucoid, or rough) are stages in an orderly predetermined life-cycle in which the expression of each stage is dependent on the environmental conditions. Hadley has stated that "each culture phase represents a stage in the development of the bacterial individual whose span of life extends from the gonidium (or similar reproductive entity) to the reproductively mature rough-phase culture. On these grounds, the bacterial individuum should not be conceived of as a single cell, but as the entire range of successive culture development from gonidium to mature form." In general, these ideas failed to survive under the impact of critical evidence[10]. It is now believed that the only remaining aspects of bacterial variation possibly involving phenomena akin to stages in a regular life-cycle are the occurrence of L-forms and the changes in cellular morphology and mode of reproduction as reported in some cytological studies (see Chapter 3).

Mutation and selection

Prior to the days of systematic research in bacterial genetics a number of bacteriologists suggested that many variational aspects observed among bacteria might be due to mutation and selection. Such suggestions were based on analogy with genetic information obtained in studies with plants and animals, rather than on critical experimental evidence. As early as 1901, soon after DeVries advanced the mutation theory for higher organisms, Beijerinck[11] suggested that phenomena of the kind which were later termed "dissociation" may be due to mutations. Other early supporters of the mutation and selection concept in bacteriology were Neisser[12], Massini[13], Cole and Wright[14], and, with certain reservations, Eisenberg[15], and van Loghem[16]. In addition, Lewis[17], Lindegren[18],

[10] See Braun, W., Bact. Rev., *11*:75, 1947, for a review of such evidence.

[11] Beijerinck, M. W., Versl. Afd. Natuurkunde Kon. Akad. Wetensch. Amsterdam, *9*:310, 1901; Folia Microbiologica, *1*:4, 1912.

[12] Neisser, L. M., Zentr. Bakt. Parasitenk. Infek. I. Ref., *38*:98, 1906.

[13] Massini, R., Arch. Hyg., *61*:250, 1907.

[14] Cole, L. J. and Wright, W. H., J. Inf. Dis., *19*:209, 1916.

[15] Eisenberg, P., Zentr. Bakt. Parasitenk. Infek. I. Orig., *73*:449, 1914.

[16] van Loghem, J. J., Z. Hyg. Infektionskrankh., *110*:382, 1929.

[17] Lewis, I. M., J. Bact., *28*:619, 1934.

[18] Lindegren, C. C., Zentr. Bakt. Parasitenk. Infek. II, *93*:113, 1935.

Reed[19], Reimann[20], Nyberg[21], Seiffert[22], Deskowitz[23], Parr and Robbins[24], were prominent among the supporters of the mutation concept whose publications preceded those which will be discussed in the following chapters.

[19] Reed, G. B., J. Bact., *34:*255, 1937.
[20] Reimann, H. A., J. Bact., *33:*513, 1937.
[21] Nyberg, C., Mitteilung. Zentr. Bakt. Parasitenk. Infek. I, *142:*178, 1938.
[22] Seiffert, W., Zentr. Bakt. Parasitenk. Infek. I. Ref., *121:*143, 1936.
[23] Deskowitz, M. W., J. Bact., *33:*349, 1937.
[24] Parr, L. W. and Robbins, M. L., J. Bact., *43:*661, 1941.

CHAPTER 3

The Cytological Basis of Bacterial Genetics

In chapter 1 it was shown that cytological studies with higher organisms have furnished geneticists with a material basis for genetic phenomena which complemented and in many respects extended the information obtained in breeding and mutation studies. Therefore, in analyzing genetic phenomena in bacteria the question arises immediately whether cellular structures and cytological processes comparable to those in higher organisms are also present in bacteria. It is difficult to answer this question wholly in the affirmative, since bacterial genetics has not yet advanced to a stage where it is possible to correlate experimental and cytological information to the extent that has been achieved in higher organisms. Many of the cytological observations on bacteria relevant to problems of variation are still controversial. Nevertheless, some pertinent facts have emerged[1, 2, 3] and their review is appropriate at this point.

Nuclear bodies

Since studies with higher organisms demonstrated that most of the determinants of hereditary characteristics are transmitted through nuclear components, it became important to ascertain whether similar organized structures exist in bacteria. Early cytological observations with bacteria were controversial in this respect, but more recent improvements of technique have verified the exist-

[1] Knaysi, G., *Elements of Bacterial Cytology*, 2nd Ed., Comstock Publ. Co., Ithaca, N. Y., 1951.

[2] Bisset, K. A., *The Cytology and Life History of Bacteria*, Williams and Wilkins Co., Baltimore, 1950; *also* Cold Spring Harbor Symp. Quant. Biol., *16*:373, 1951.

[3] Robinow, C. F., in *The Bacterial Cell*, by R. J. Dubos, Harvard University Press, Cambridge, Mass., 1945.

ence of nuclei in bacterial cells. An organized nucleus in bacterial cells was described by Meyer[4] as early as 1897 and was recognized repeatedly thereafter.[5] Remarkable progress in the demonstration of nuclei was achieved following the recognition that the ribonucleic acid of the basophilic bacterial cytoplasm obscured the staining reactions of the nucleus. Following the removal of ribonucleic acid by either mild hydrolysis, ribonuclease or preliminary

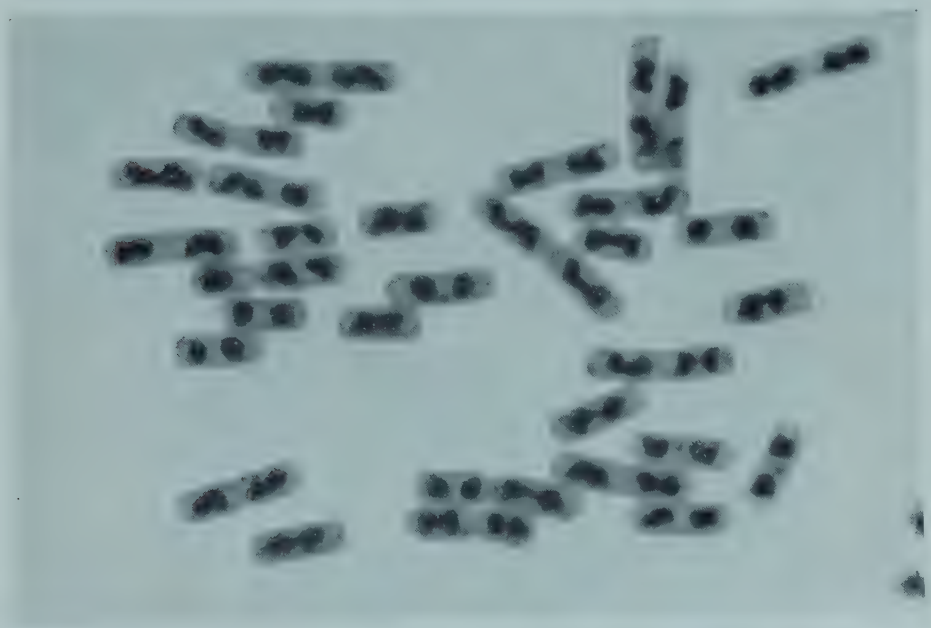


Fig. 13. Chromatinic structures in *Bacillus cereus* cells. Fixed with osmium vapor, stained by acid-Giemsa. Photographed mounted in water. Magnified 3000 times. (C. Robinow.)

partial starvation of cells, the nucleus can be stained by a number of dyes known to have a specific affinity for nuclei of higher organisms or by means of the Feulgen reaction which is specific for aldehydes, including those formed by desoxyribose nucleic acid (DNA) following hydrolysis. The DNA nature of the bodies recognized as nuclei has also been confirmed by ultraviolet absorption. Robinow[6] popularized a technique first described by Pickarski,[7] namely the use of Giemsa's solution for staining nuclei following fixation with osmium tetraoxide vapor and treatment with 1N HCl at 60° C. With the help of this technique, Robinow,[6] Bisset,[2] Dela-

[4] See Meyer, A., *Die Zelle der Bakterien*, Gustav Fischer, Jena, 1912.
[5] See Knaysi's book for a historical review.
[6] Robinow, C. F., Proc. Roy. Soc. B., *130*:299, 1942.
[7] Pickarski, G., Arch. Microbiol., *8*:428, 1937.

porte,[8] and others demonstrated deeply stained, often vesicular, bodies within the bacterial cell (Fig. 13). These bodies, consisting primarily of desoxyribonucleoprotein (DNA-protein) have been described as rod-shaped, usually forming a double rod, in actively growing cells of Eubacteria; they appear spheric in most cocci,

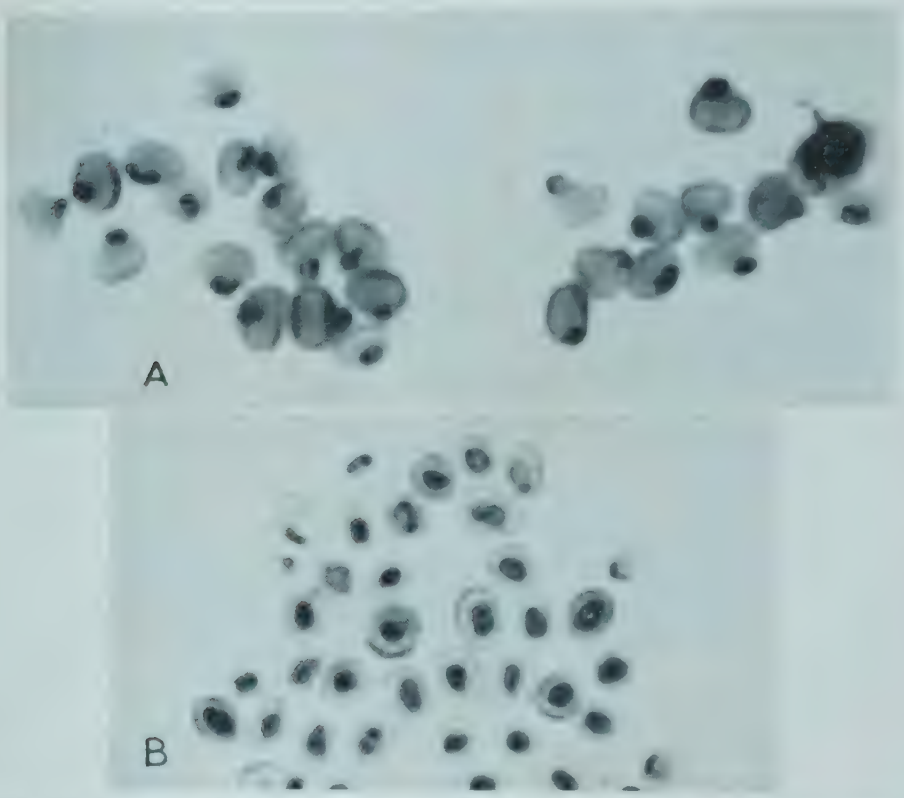


Fig. 14. Spores of *B. megatherium*. *A*, Resting spores of *B. megatherium* from a 4-day culture on potato extract agar. The nuclei are at the periphery. *B*, Spores from the same culture as those in *A*. Fixed after 10 minutes on heart infusion agar at 37° C. In most of the spores the nucleus is now in the interior of the cell and appears ring-shaped in optical section. Both preparations fixed with osmium vapor, hydrolyzed with 1N HCl at 60° C. and stained with Delamater's SO_2-thionine. Photographed mounted in water. Magnified 3600 times. (C. Robinow.)

acid-fast and diphtheroid bacteria. The double rod or paired appearance of many nuclei is probably a reflection of the distribution of chromatinic, intranuclear elements, i.e., deeply stained elements rich in DNA-protein, during nuclear division (see below). A nuclear body also has been demonstrated in spores (Fig. 14). When resting spores are hydrolyzed prior to staining, a rearrangement of the spore

[8] Delaporte, B., Advances in Genetics, *3*:1, 1950.

contents occurs which results in the appearance of peripheral and even of more or less completely extruded nuclei (Fig. 14 A). However, this response to hydrolysis is radically changed as soon as germination has been initiated. The chromatin is now found forming the shell of a spherical structure in the interior of the spore and in optical sections it appears arranged in the shape of a ring[9] (Fig. 14 B).

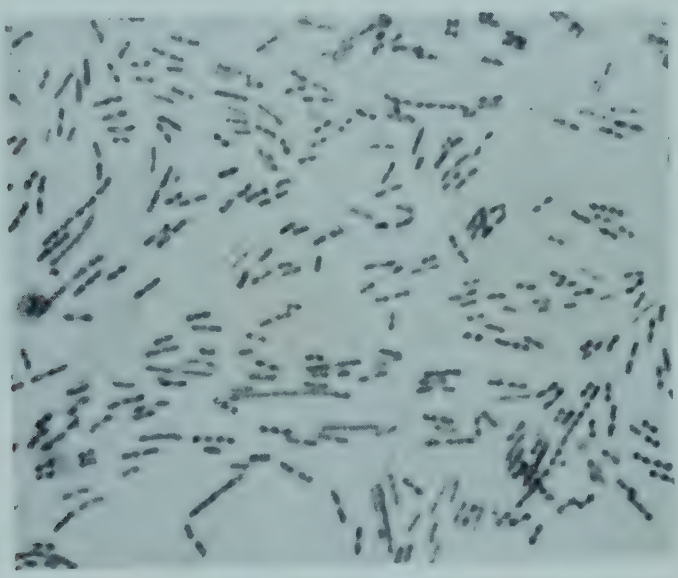


Fig. 15. Cells of *E. coli*, B 'r (radiation-resistant), streaked on nutrient agar following 25 ergs mm^2 UV-irradiation and incubated for 3 hours at 37° C. Stained by DeLamater's method.[10] Magnified 1250 times. (V. Bryson.)

The number of nuclei per vegetative cell may vary. Thus, cocci are usually uninuclear, whereas cells of rodlike bacteria are frequently multinuclear. The latter may contain two nuclei or multiples of two, particularly during stages of active growth (see Fig. 8). The multinuclear condition is caused by a lack of synchronization between the rate of growth and the rate of cell division. The rate of nuclear division appears to be proportional to the rate of growth. Therefore, in young, actively growing cultures, multinuclear cells are apt to predominate because the nucleus may divide several times before the cell divides. In older cultures the majority of cells may become uninuclear. Many inhibitory conditions, such as exposure to ultraviolet radiation or penicillin, also cause the formation of multinuclear cells, since such conditions can interfere with cell division without immediate effects upon nuclear

[9] Robinow, C. F., personal communication.

division. Thus filamentous cells containing many nuclei are formed under these adverse conditions (Figs. 15 and 16). The existence of more than one nucleus in many bacterial cells is an important factor which must be recognized in genetic studies (see p. 75). The effects of changes occurring in a haploid nucleus can be masked by the presence of one or more unaltered nuclei in the same cell, similar to the manner in which the presence of one unaltered chro-

Fig. 16. Cells of *E. coli*, B (radiation-sensitive), treated in the same manner as stated in Fig. 15. Magnified 1250 times. (V. Bryson.)

mosome can mask the phenotypic effects of a recessive change in the other member of a pair of homologous chromosomes in diploid higher organisms.

Resting cells of rodlike bacteria and spores are usually described as being uninuclear. According to Bisset,[2] cells of the so-called smooth bacilli usually contain a pair of nuclei, whereas cells of rough bacilli are uninuclear (see Fig. 20).

The occurrence of fusion between the individual nuclei of multinuclear cells, *autogamy*, has been claimed to occur in bacteria, especially during maturation of the spore.[2] The interpretation of the cytological evidence is still controversial and the role of possible autogamous processes in bacteria is difficult to assess in the absence of supporting genetic data.

In the older cytologic literature it is usually stated that the bacterial nucleus divides longitudinally without revealing any special internal structure. However, newer techniques have uncovered or-

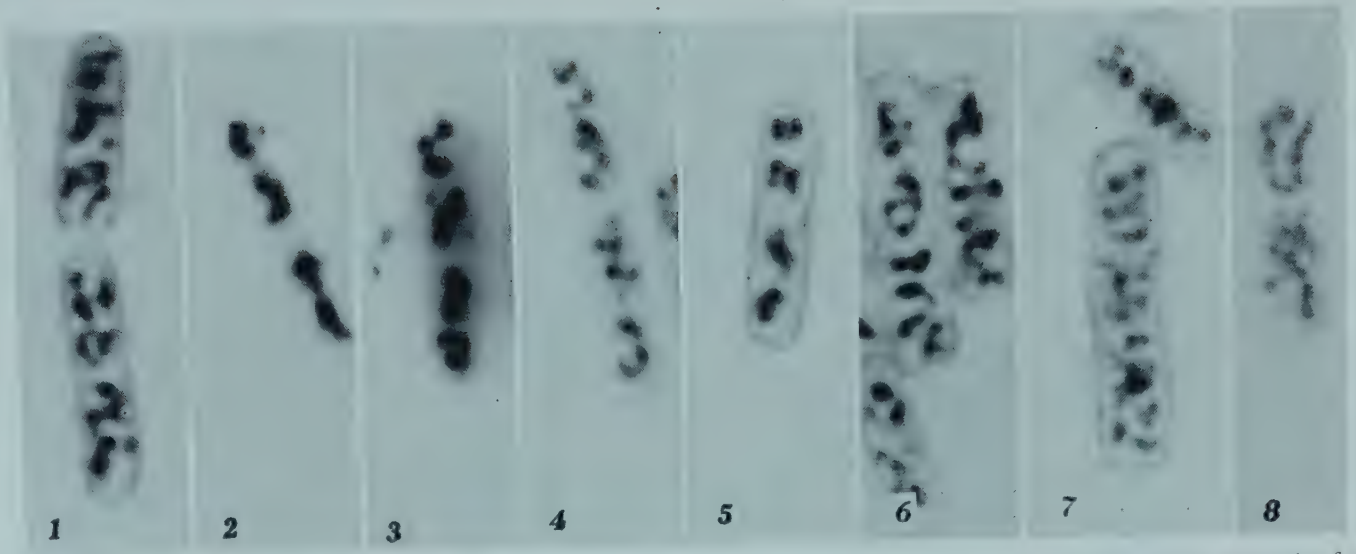


Fig. 17. Mitotic cycle in *Bacillus megatherium*. Cells fixed and stained according to the method of DeLamater.[10] Magnified 4450 times. *1*, Early prophase: Chromosomes contracting. Three chromosomes visible in lower nucleus of upper cell. *2*, Topmost nucleus shows late prophase with chromosomes condensed and centriole visible. *3*, Second nucleus from top shows metaphase spindle with two centrioles at poles. Upper nucleus shows polar view with three chromosomes. *4*, Telophase: Chromosomes separating to poles. *5*, Late telophase or early anaphase: Chromosomes separated. Second nucleus from top shows three chromosomes. *6*, Early interphase: Nuclear membrane re-formed and chromosomes elongated in second nucleus from top. *7*, Upper nucleus in larger cell shows late interphase with nucleus as discrete body with distinct nuclear membrane and chromosomes in long threads. Chromosomes cut in optical sections. *8*, Late interphase: Chromosomes as long, beaded threads. (Photos and legends by E. D. DeLamater.)

ganized chromatinic structures which indicate that a true mitotic process occurs, similar to that observed in higher organisms.

Intranuclear structures

Although mitotic processes displaying chromosome-like bodies have occasionally been reported in the older literature, their con-

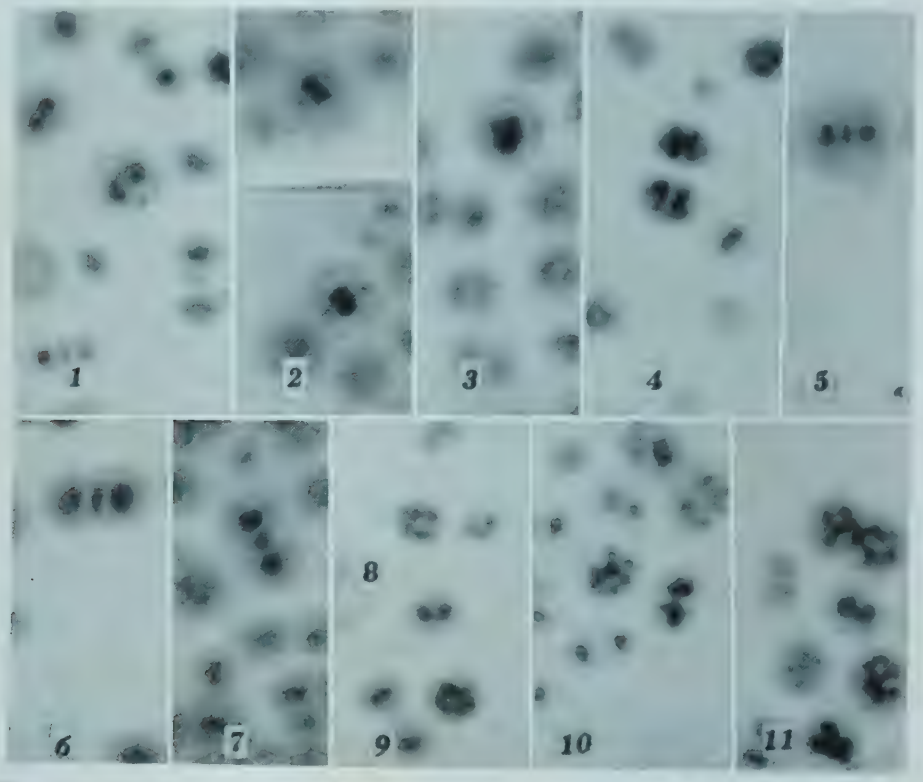


Fig. 18. Mitotic cycle in *Micrococcus cryophilus*. Cells fixed and stained according to the method of DeLamater.[19] Magnified 4450 times. *1*, Late prophase: Two compact chromosomes with single centriole. *2*, Metaphase spindle: Two compact chromosomes on metaphase plate between centrioles. *3*, Anaphase: Early stage in separation of chromosomes, centrioles persist. *4*, Telophase: Chromosomes migrating to poles. *5*, Chromosomes in compact masses in two daughter nuclei. Centriole not visible. Cell plate has developed between sister nuclei. *6–7*, Later stage in cell plate formation. Chromosomes still dense in early interphase. Other cells show later interphase stages with chromosomes elongating. *8–9*, Later interphase nuclei. Chromosomes as tangled threads. Metaphase spindle in upper right hand corner. *10*, Late interphase: Chromosomes as threads. Central nucleolus visible. *11*, Early prophase: Chromosomes contracting. (Photos and legends by E. D. DeLamater.)

sistent demonstration in all of the several species so far investigated has become possible by the application of a freezing-dehydration

technique employed by DeLamater and co-workers.[10] Following fixation with osmium tetraoxide and hydrolysis with 1 N HCl at 60° C, the material is stained with either (1) 0.25 per cent thionine containing a drop of thionyl chloride or (2) 0.25 per cent azure A containing small amounts of thionyl chloride. After the preparations are stained, they are immediately placed in absolute alcohol at −50° C, where they are kept for 12 hours. Dehydration then proceeds gradually. Inspection of such preparations has revealed structural details and processes akin to mitosis in higher organisms. Figures 17 and 18 show different stages in the mitotic cycle of *Bacillus megatherium* and *Micrococcus cryophilus*. Similar con-

Fig. 19. Diagram illustrating how mitotic divisions in a multinucleate cell may occur in different planes. Each one of the four dividing nuclei in above sketch is assumed to be in late metaphase.

figurations also have been observed in studies with *Caryophanon latum,* a large gram-negative bacillus, *Micrococcus albus, Bacillus cereus,* and *Escherichia coli*. Since in multinuclear cells, e.g., in rod-shaped bacteria, mitotic divisions may take place in all of the nuclei simultaneously, it is important to recognize that the plane and axis of division may differ between individual nuclei. Thus within a single cell one of the divisional figures may be observed as from above, whereas another one may offer a side view (Fig. 19).

Attempts to count the number of chromosomes during mitosis[10] have led DeLamater to suggest that the two types of cocci studied contain two chromosomes, and *Bacillus cereus, Bacillus megatherium,* as well as *E. coli,*[11] three chromosomes per vegetative nucleus. These estimates, however, are not considered final.

Bacteriologists are almost unanimous in their conclusion that the majority of bacterial cells and spores are haploid. As we shall see later, genetic evidence supports this conclusion. However, it has been claimed by some that vegetative cells of spore-formers may be

[10] DeLamater, E. D., Cold Spring Harbor Symp. Quant. Biol., *16*:381, 1951.
[11] DeLamater, E. D., Genetics, *37*:576, 1952.

diploid prior to spore formation and that a reduction division precedes the maturation of the spore. Similarly, Delaporte[9] claims that only a fraction of the nuclear elements of the vegetative cell is incorporated into the spore. In contrast, DeLamater's studies on *B. megatherium* have indicated that the division leading to the formation of the spore is typically mitotic and not meiotic, i.e., it is not a reduction division.

We have already referred to the claims that fusion of nuclei, presumably leading to diploid nuclei, may occur in various species. In addition, phenomena involving sexual fusion between cells, leading to diploid cells, have been reported repeatedly,[5, 10] but these reports, including the claims for the occurrence of complex life-cycles, cannot be evaluated properly until supporting evidence has been obtained from genetic studies. So far only one case of diploidy is supported by the results of experimental genetic studies. This diploid stage was detected in certain strains of *E. coli* (see p. 193).

Polyploidy has not yet been detected among bacteria. However, cases such as the formation of giant cells of *P. pestis* following treatment with camphor,[12] a known polyploidizing agent, suggest the possible occurrence of such a phenomenon.

The cytologic observations, indicating the existence of true chromosomes in bacteria, are also supported by the finding that nucleic acid-containing elements with a coiled structure, typical of the chromosomes of higher organisms, can be separated from broken *E. coli* cells following differential centrifugation.[13]

Reproductive mechanisms

In discussing the bacterial nucleus we have already referred to several reproductive mechanisms in bacteria: (1) vegetative, asexual reproduction by simple cell division following nuclear division by mitosis; (2) spore formation, now also suspected to be of vegetative nature at least in some cases; and (3) suspected sexual reproduction involving either nuclear fusion within cells, or cellular fusion followed by nuclear fusion. In addition, another phenomenon of potential genetic significance deserves mention, namely the so-called "L-forms" of bacteria.[14, 15] It has been observed that some-

[12] Won, W. D., J. Bact., *60*:102, 1950.

[13] Marshak, A., Proc. Nat. Acad. Sci., *37*:38, 1951; Exp. Cell Res., *2*:243, 1951.

[14] Dienes, L. and Weinberger, H. J., Bact. Rev., *15*:245, 1951.

[15] Klieneberger-Nobel, E., Bact. Rev., *15*:103, 1951.

times spontaneously, but more frequently after exposure to adverse conditions such as penicillin, phage, or antibodies, bacillary forms of various gram-negative and gram-positive species may give rise to tiny chromatin-rich granules and large swollen amorphous bodies. These elements develop into tiny, so-called "L-type" colonies on solid media. It has been claimed that the large bodies develop from the fusion of the tiny granules[15]; Dienes[14] has stated that

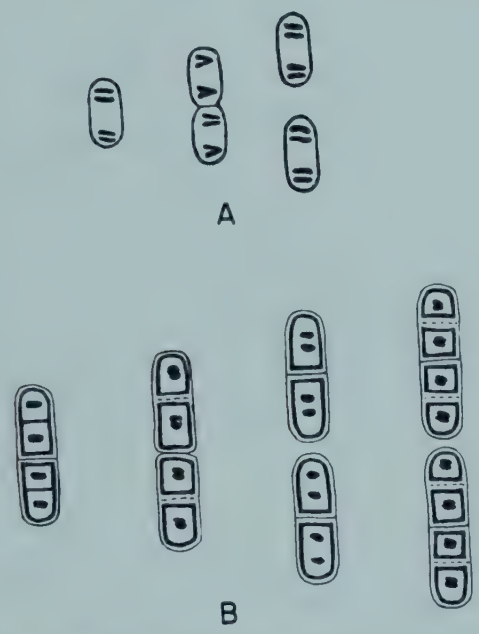


Fig. 20. Nuclear and cell division in smooth and rough bacteria according to Bisset. *A*, Smooth bacterium; *B*, rough bacillus. The cell wall is represented by the fine line, dotted where new cross walls are in process of secretion. The cell membrane is represented by the thick line. (Reproduced by courtesy of the Journal of General Microbiology.)

the large bodies can give rise to granular elements. Reversion from such L-forms to typical bacillary forms has been reported in a number of cases. Klieneberger-Nobel[15] has shown that the L-type granules of *Streptobacillus moniliformis* can pass filters with a pore-size of 400 mμ and subsequently develop into L-type colonies, whereas the typical bacillary form of this species does not pass through filters with a pore-size of less than 800 mμ. This observation has led her to suggest that L-forms may be responsible for the reports on filterable forms of various bacteria. However, the manner in which such observations can be integrated with known genetic phenomena in bacteria is still largely a matter of speculation.

Still another reproduction phenomenon of potential interest to

problems of bacterial genetics concerns certain modes of cell division during asexual reproduction. Bisset[2] has presented evidence indicating that cells of the so-called "smooth" type of Eubacteria (see also p. 105) are usually unicellular, having a tendency to separate completely at time of cell division. In contrast "rough" type cells are described as usually multicellular, usually four-celled elements, in which a cell wall and membranous septa separate the individual cells which have a tendency to remain attached at the point of division (Fig. 20). This differential mode of division, aside from being responsible for the different types of colonies formed by "smooth" and "rough" bacteria (see Fig. 32), could be of critical importance in any genetic study designed to test the behavior of the progeny of a single cell. In the case of multicellular rough elements, it would be impossible to isolate the progeny of a single cell, i.e., a clone, merely by picking a single colony, since such a colony would be likely to have developed from several sister cells. Similar considerations apply to the isolation of clones from species where cells do not tend to occur singly; for example, in the case of staphylococci or streptococci.

Bacterial cytogenetics

The foregoing discussion has shown that, except for size, the cytology of bacteria does not differ essentially from structures and phenomena known in larger organisms. It is now established that bacteria possess nuclear structures which are distributed in a regular fashion to daughter cells, thus insuring a continuity of nuclear material in the same manner as has been recorded for higher organisms. It follows that a material basis for the hereditary determinants in bacteria similar to that established for higher organisms does exist. However, a systematic attempt to correlate cytologic structures and phenomena with genetic processes in bacteria, i.e., the development of the field of bacterial cytogenetics, is only now in the process of evolving (see p. 76). It can be expected that a full integration of the newer techniques of bacterial cytology and the type of genetic information which we shall discuss in the following chapters will result in rapid progress in this field.

CHAPTER 4

Mutation

THE EXPERIMENTAL APPROACH TO BACTERIAL GENETICS HAS BEEN concerned largely with the problem of bacterial mutability, its origin, cause and manifestation, and its effect on changes in bacterial populations. One of the major results of these studies has been the demonstration that hereditary changes in bacterial cells can be caused by *mutations*. As in higher organisms, mutations can be defined as spontaneous, undirected changes that may occur in the nuclear determinants of hereditary characteristics. A mutation is a rare event which usually occurs at a rate of between 1×10^{-4} and 1×10^{-10} per bacterium per generation. This means that, depending on the *mutation rate*, one bacterial cell in 10,000 or one cell in 10 billions is apt to be a mutant. A mutant cell is a cell carrying a mutated gene and as such will give rise to mutant clones. Naturally, only a few of the spontaneously occurring mutations are usually detected; unless the environment is suitable for the establishment of the mutant in the population in which it has occurred, the mutant cell will have little survival value and remain undetected. Even under standard environmental conditions, mutation rates differ for different characteristics and they may also differ for similar characteristics between different strains. However, mutation rates are relatively constant for any given gene in a given strain. For example, the mutation rate from streptomycin sensitivity to streptomycin resistance in strain B of *E. coli* has been found to be 1×10^{-10} in numerous tests conducted at different times. Certain environmental agents are capable of increasing spontaneous mutation rates significantly and such agents are called mutagens (see Chapter 5).

It is generally agreed that mutations are entirely undirected, i.e., mutants occur independently of the environment to which they may be better adapted. Thus, mutations to bacteriophage or streptomycin resistance can occur in bacterial populations growing in the absence of phage or streptomycin. However, in order to detect such

mutants it usually becomes necessary to submit the populations to the influence of phage or streptomycin. Under such conditions the susceptible parent cells fail to grow whereas the resistant mutants become detectable through their ability to grow in this selective environment. Such screening procedures have been extremely valuable in the demonstration of the occurrence of spontaneous undirected mutations among bacteria.

EVIDENCE FOR THE OCCURRENCE OF MUTATIONS IN BACTERIA

Although intensive studies of higher organisms had familiarized geneticists with mutations over a period of almost 50 years, a clear experimental demonstration of the occurrence of spontaneous, undirected mutations in bacteria was lacking until 1943 when Luria and Delbrück[1] reported their "fluctuation test." Since their test is based on statistical considerations and is somewhat indirect in nature, we shall first review a simpler and more direct experimental proof which was contributed by Newcombe in 1949.

Newcombe's spreading experiment

Newcombe[2] designed an experiment with the phage-susceptible B r strain of *E. coli* which would permit a differentiation between the possibility that phage-resistant variants arise due to the exposure to the specific phage-containing environment (adaptation hypothesis) and the possibility that phage-resistant variants arise spontaneously during normal growth in the absence of phage (spontaneous mutation hypothesis). Under the latter hypothesis the addition of phage would merely select the resistant cells already present. He plated phage-susceptible cells on agar and incubated the plates for several hours during which time a limited population increase, i.e., growth into microcolonies, took place. On alternate plates, the cells were then redistributed over the agar surface by spreading with 0.1 ml of saline. The remaining plates were left undisturbed. All plates were then sprayed with phage T_1, reincubated, and the number of colonies that developed from the surviving resistant cells were counted. If the resistant variants developed due to contact with the phage (adaptation hypothesis) it should make no difference whether or not plates were spread prior to spraying with phage; all bacteria present at the end of the initial growth period

[1] Luria, S. E. and Delbrück, M., Genetics, *28*:491, 1943.
[2] Newcombe, H. B., Nature, *164*:150, 1949.

would be assumed to be phage-susceptible and spreading would only result in the redistribution of members of a homogeneous population. Therefore, according to the adaptation hypothesis no significant differences in final colony counts (colonies developing from phage-resistant cells) would be expected between spread and unspread plates. In contrast, if phage-resistant variants arise spontaneously in the absence of phage (mutation hypothesis), both resistant and susceptible cells should be present after the initial growth period, provided a sufficient population size had been

Fig. 21. Newcombe's spreading experiment. 5.7 x 10^6 bacteria were plated, the plates were incubated for six hours, those in the upper part of the picture were then respread with 0.1 ml sterile saline, and all were sprayed with phage T_1. The population increase prior to respreading and phage exposure was in the vicinity of 5000 fold. (H. B. Newcombe.)

reached at the time of phage-spraying. Depending on whether such mutations occurred during the last or previous generations a microcolony may contain from one to several mutant cells since one mutant cell appearing in prior generations could have given rise to mutant clones or mutant clusters within single colonies. Spreading would break up such mutant clusters and each individual mutant cell would then give rise to a resistant colony. However, on unspread plates each cluster within each individual microcolony would give rise to only one single resistant colony. Thus, if the resistant cells arose by spontaneous mutation, prior to contact with phage, more resistant colonies should be found on spread plates than on unspread plates. This is exactly what was observed (see Figure 21 and Table 1). This experiment thus confirms that phage-resistant

FOOD TECHNOLOGICAL RESEARCH INSTITUTE
LIBRARY

variants arise by a spontaneous change (mutation) prior to exposure to phage. The phage acts merely as a selective agent permitting the detection of the few phage-resistant mutants present in the population.

TABLE 1. *Resistant colonies developing after spraying agar plate cultures of* E. coli *with phage* T_1, *showing the effect of redistribution by spreading prior to spraying.*

(From Newcombe, H. B., Nature, *164*:150, 1949)

INCUB. (HR.)	3		4		5		6	
Bact. plated	5.1×10^4		5.1×10^4		5.1×10^4		5.1×10^4	
End No. bact.*	1.7×10^6		2.3×10^7		2.6×10^8		2.8×10^9	
Factor increase	33		480		5,100		54,900	
Resist. colonies	unsp.	sp.	unsp.	sp.	unsp.	sp.	unsp.	sp.
replicate test 1	0	0	0	0	5	194	46	2,254
2	0	0	3	0	3	14	25	1,434
3	0	1	0	6	4	16	45	3,294
4	0	0	2	0	8	13	49	3,719
5	0	0	1	0	2	4	26	1,538
6	0	1	2	2	6	112	49	399
Total	0	2	8	8	28	353	240	12,638

* Estimated by washing and assay; averages of three independent determinations. unsp. = unspread; sp. = spread.

Similar results were obtained when the spreading test was used in a study with streptomycin-resistant variants.[3]

The fluctuation test

Luria and Delbrück[1] submitted experimental evidence for the spontaneous occurrence of bacterial variants several years prior to the publication of Newcombe's spreading experiment. Their evidence was based on the results of the fluctuation test (Fig. 22) which is performed as follows: A series of tubes, usually containing 0.2 or 0.5 ml of liquid medium, is inoculated with a small number of phage-sensitive cells. These cultures are incubated, without phage, until a certain population size is reached. The number of phage-resistant mutants in each tube is then determined following exposure of the cultures to phage, usually by pouring the contents

[1] Bornschein, H., Dittrich, W. and Höhne, G., Naturwissensch., *38*:383, 1951.

of each tube into an agar plate containing phage. The results obtained from such a *series of similar cultures* can then be compared with the results of a series of samples taken from *one culture* started with a similar concentration of cells per ml and permitted to attain a similar population size per ml. The principle of the test is this: If resistant variants arise because of contact with the phage (adaptation) it should not make any difference whether populations exposed to phage came from a series of similar cultures or from one culture. At the time of exposure to phage, all cells would pre-

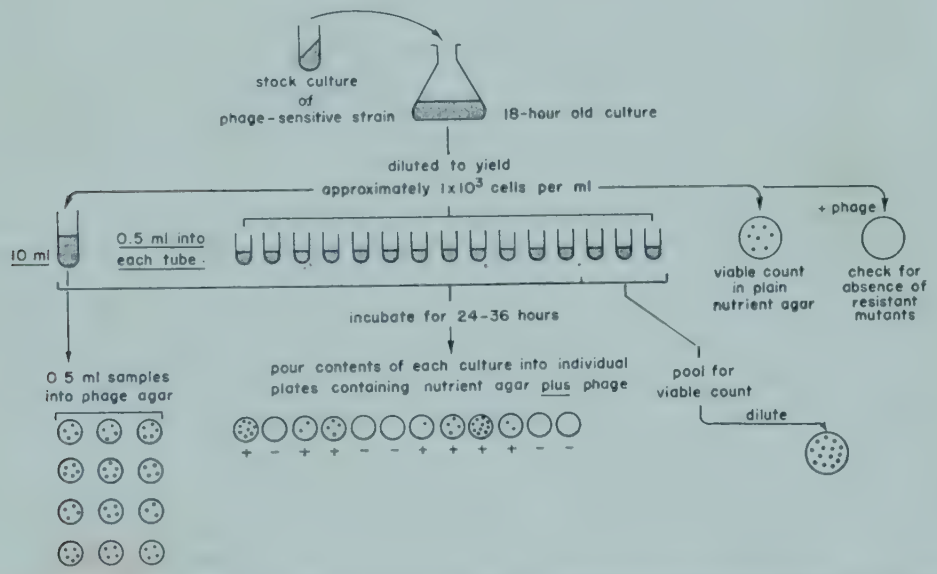


Fig. 22. Diagram of the fluctuation test. In above example a test for phage-resistant mutants of *E. coli* is illustrated.

sumably be sensitive and the number of resistant bacteria obtained from a series of similar samples should show only the fluctuation due to sampling error (Poisson distribution) regardless of whether they came from a series of parallel cultures or from replicate sampling of a single culture. On the other hand, if resistant bacteria arise spontaneously prior to the exposure to phage, a series of similar cultures will yield results different from those obtained with a series of samples from one culture. The reason is again the clonal distribution of mutants discussed above in connection with Newcombe's experiment. Parallel cultures may differ not only in the number of mutations which have occurred in each culture but in the time at which these took place during the culture's growth. Thus, if one mutation occurred just prior to exposure to the phage a culture would display only one resistant cell; however, if a similar

mutation occurred several generations earlier it would have given rise to a number of resistant descendants (clone). Hence, even if equal numbers of mutations occurred in parallel cultures, the number of phage-resistant cells at the time of exposure to phage would be expected to differ considerably among these cultures. Numerous

TABLE 2. *Number of bacteria* (E. coli) *resistant to a concentration of 5 units of streptomycin per ml of agar medium in samples taken from a series of independent cultures and similar samples taken from a single culture which assayed 1.3 x 10^8 bacteria per ml.*

(From Demerec, M., J. Bact., *56*:63, 1948)

SAMPLES FROM INDEPENDENT CULTURES				SAMPLES FROM SINGLE CULTURE			
Culture no.	No. of resistant bacteria	Culture no.	No. of resistant bacteria	Sample no.	No. of resistant bacteria	Sample no.	No. of resistant bacteria
1	67	11	56	1	142	11	110
2	159	12	91	2	155	12	125
3	135	13	123	3	132	13	135
4	291	14	97	4	123	14	121
5	75	15	48	5	140	15	112
6	117	16	52	6	146		
7	73	17	54	7	141		
8	129	18	89	8	137		
9	86	19	111	9	128		
10	101	20	164	10	121		
Average			105.9	Average			131.2
Variance			2913.9	Variance			151.1
Chi-square			550.3	Chi-square			17.3
P much less than			0.001	P			0.26

tests have confirmed that this is the case; the fluctuation in the number of phage-resistant variants obtained in tests on a series of parallel cultures is significantly greater than that obtained in tests on a series of samples from one culture (Table 2 and Fig. 22). These results clearly support the hypothesis of spontaneous mutation. Similar results were obtained when the origin of resistance to various antibiotics, resistance to radiation, changes in fermentative abilities, or changes in growth requirements were tested with the help of the fluctuation test.[4]

[4] See Table 3 for some representative references.

Lederberg's indirect selection test

One of the important features of the above techniques is the use of selective conditions (e.g., phage, antibiotic) which prevent the multiplication of the parent cells but allow the growth of the mutants, thus permitting the detection of a few mutants among the many parent cells. Since most mutants arise at the rate of 1 in 10^8 or even 1 in 10^{10} cells this elimination of the parent cells is usually required for the detection of mutants. Yet the most direct method of demonstrating the undirected, spontaneous origin of bacterial mutants would consist of the isolation of mutants in the absence of the environmental conditions that favor their establishment. This ideal situation has been achieved in tests employing the replica plating technique.

The Lederbergs[5] have described a very simple method that permits the transfer or replica plating of bacterial growth from one initial plate to corresponding sites on a series of other plates. This transfer is accomplished with the help of velveteen which is placed, nap up, on a cylindrical wood or metal support having a diameter slightly less than that of a Petri plate. A rubber band or metal flange is used to hold the fabric firmly in place. The colonial growth which has developed on the initial agar plate is then transferred to the velveteen by inverting this plate onto the fabric. The imprinted fabric now can serve as the master pattern for the inoculation of other plates, each thread in the pile fabric acting as an inoculating needle when sterile agar plates are inverted on the velveteen. Replica-inocula can be made from one imprinted velveteen sample onto numerous plates, containing different media if so desired. The resulting growth on all these plates will be at corresponding sites (Fig. 23) since members of each clone that developed on the initial plate are distributed to the new plates without disturbance of their spatial relationships.[6] This has permitted a confirmation of previous observations regarding the clonal occurrence of bacterial variants which, as explained above, indicates that such variants arise spontaneously and independently of a specific environment. The growth from a phage-susceptible population which had developed on plain nutrient agar was imprinted on velveteen

[5] Lederberg, J. and Lederberg, E. M., J. Bact., *63*:399, 1952.

[6] The replica plating technique is useful for many purposes, including rapid detection of biochemical mutants (Fig. 22), classification of fermentation reactions, and determination of spectra of antibiotic sensitivity.

and serial replicas were then transferred to several plates containing nutrient agar *plus* phage. Colonies consisting of phage-resistant cells were found to develop on identical sites on each replica plate. This indicated that the resistant cells transferred to the phage plates

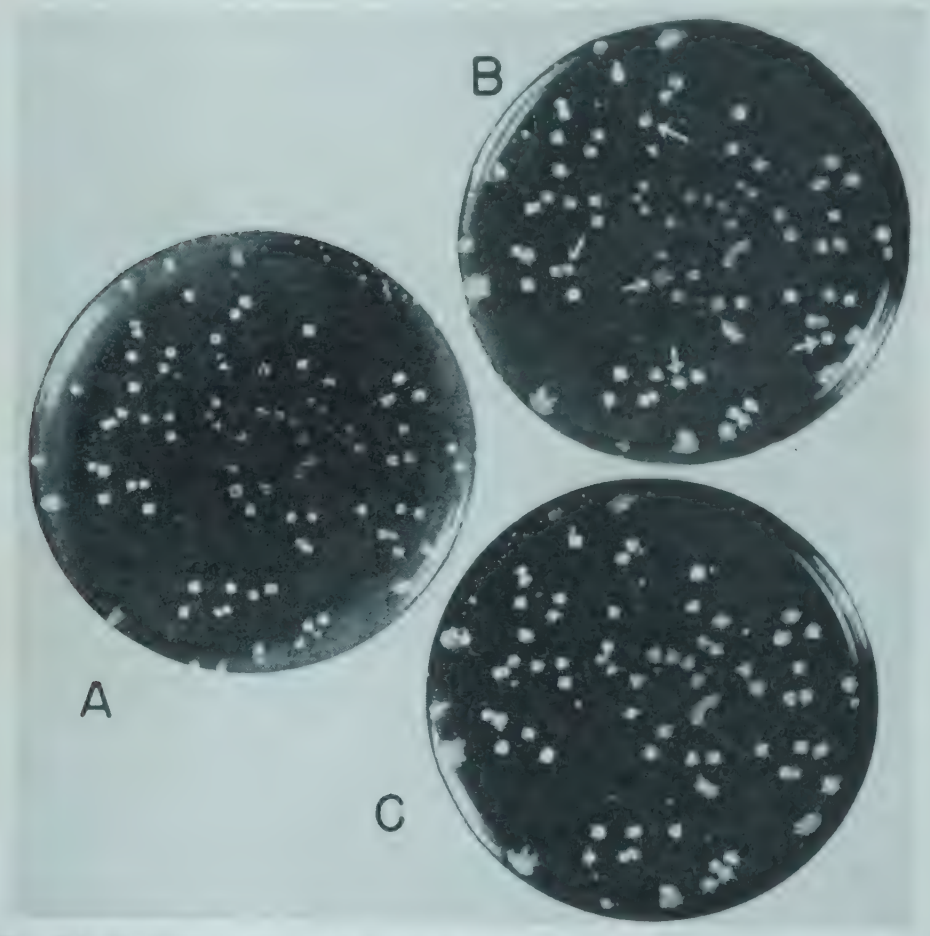


Fig. 23. Replica plating. *A*, Initial plate; *B* and *C*, replicas. This picture also illustrates the use of replica plating for the isolation of auxotrophic colonies. *A* and *B* are plates with complete agar medium; *C* is a replica on minimal agar. The arrows designate the auxotrophic colonies which fail to grow on minimal medium. (J. and E. M. Lederberg.)

were derived from small clones of resistant mutants already present at corresponding sites on the initial plain agar plate. Identical results were obtained in the case of streptomycin-resistant mutants.

The isolation of pure cultures of resistant mutants without exposure to any selective, or possibly inductive, agents was accomplished by replica plating in the following manner (Fig. 24): The growth from a phage-susceptible inoculum on plain agar was imprinted on velveteen which was then used for inoculating both

plain and phage-containing nutrient agar plates. Subsequently, the area of growth on a plain plate corresponding to the site where a phage-resistant colony developed on "phage agar" was picked. Since it could be assumed that this area not only contained one or a few phage-resistant cells but was still highly "contaminated" with unchanged phage-susceptible cells, an enrichment procedure became necessary in order to obtain a pure culture of resistant cells. The isolate from the plain agar plate, therefore, was transferred to plain nutrient broth, incubated, and an aliquot from this broth culture was used for inoculating plain agar. The resulting growth, now

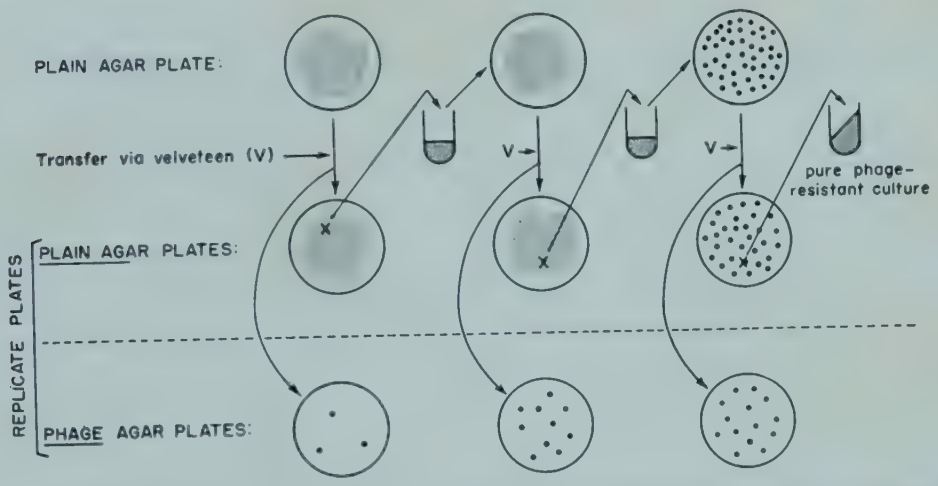


Fig. 24. Diagram illustrating the technique of indirect selection of mutants by replica plating. See text for further details.

containing a larger concentration of phage-resistant cells, was then used for a repetition of the described replica platings. This procedure was repeated four times, and each time the location of the presumably phage-resistant cells on plain agar was determined by inspection of the site of growth on replicate plates containing agar *plus* phage. Following this process of indirect selection it was possible to obtain a pure culture consisting of stable, phage-resistant cells. Streptomycin-resistant populations were isolated by the same procedure. It is important to note that such variants were isolated without altering the media in which the bacteria grew; the indirectly selected resistant populations were not exposed either to phage or streptomycin at any time. These observations thus confirm the mutational origin of these variants, i.e., they arose independently of the environment to which they are better adapted.

The isolation of mutants in the absence of the environmental

conditions that favor their establishment was also accomplished in the case of streptomycin-resistant mutants of *Micrococcus pyogenes*.[7] It was observed that in the absence of streptomycin these mutants usually grew at a faster rate than the streptomycin-sensitive parent cells. Isolation of such fast growing cells from cultures free of streptomycin and subsequent testing in streptomycin-containing media revealed that every one of these isolates yielded streptomycin-resistant clones. As in the cases analyzed by replica plating, adaptation could be ruled out conclusively because the mutants arose spontaneously and were isolated in the absence of the agent to which they proved resistant.

Studies on the mutational origin of tryptophan-independence

From a historical as well as technical standpoint Fildes and Whitaker's[8] analysis of the mutational origin of *Bact. typhosum* cells capable of growing without tryptophan is of considerable interest. Fildes was once a staunch supporter of the concept that bacterial variants arose by "training," i.e., he assumed that a certain proportion of the tryptophan-requiring cells were capable of adapting to growth without tryptophan if they were maintained in the absence of the amino acid, or, in his own words, "that the enzyme make-up of the bacteria became altered as a result of a stimulus produced by deficiency of the metabolite." In a series of experiments, published in 1944, this concept was reexamined and evidence was obtained for the mutational origin of these variants. They were shown to occur by pure chance and unrelated to the environment. It was determined that a small proportion of tryptophan-independent cells (about 1 in 10^7) is always present in normal tryptophan-requiring cultures and that their appearance is unrelated to the concentration of tryptophan in the culture. However, their ability to establish themselves during growth of the culture depends on the concentration of tryptophan. This was determined in cultures started with 10 tryptophan-requiring cells per ml and incubated at 37° C. If a large amount of tryptophan was present, the parent cells outgrew the small number of mutants present until all population increase ceased due to exhaustion of some other nutritional factor. However, if the concentration of tryptophan was limited to suboptimal amounts, mutants grew in excess of

[7] English, A. R. and McCoy, E., J. Bact., *62*:19, 1951.
[8] Fildes, P. and Whitaker, K., Brit. J. Exp. Path., *29*:240, 1948.

the parent cells until the medium was exhausted, the mutants making their appearance when a population density of approximately 1 x 10^7 per ml was reached. A partial deprivation of tryptophan,

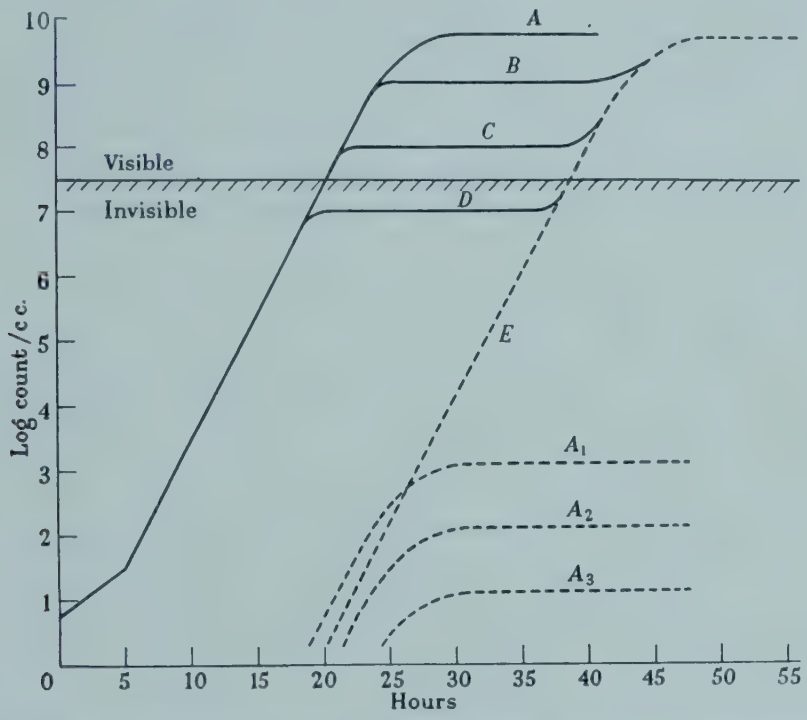


Fig. 25. Effect of tryptophan on the growth of tryptophan-independent mutants occurring in a culture of tryptophan-requiring *Bact. typhosum* cells (diagrammatic). ——— = Total viable count; - - - - - - = mutants. *A* = Peak of viable count of a culture unlimited by inadequacy of tryptophan. A_1, A_2, A_3 = Growth curves of any mutants that happen to appear in cultures like *A* when they have reached 10^7, 10^8 or 10^9 bacteria/ml, respectively. A_1, A_2, and A_3 are equivalent to that part of curve *A* which is drawn above the point of occurrence of the mutant. The available nutrients, other than tryptophan, limit the total growth of both parent and mutant cells. Both grow for the same number of generations, 9 in the case of *A* and A_1. *B* is a culture with a peak of growth of the parent type limited to 10^9 bacteria/ml by deficiency of tryptophan; *C* is another culture limited to 10^8/ml; *D* is limited to 10^7/ml. A tryptophan-independent mutant is assumed to have arisen when the parent population reached 10^7/ml. *E* represents the growth of the progeny of such mutants which overtakes the limited cultures and carries the total viable count up to the limit. Culture *D* had not grown *visibly*, and in this case the whole of the visible growth is due to mutant type cells. (From Fildes and Whitaker.[8])

by creating conditions selective for the mutant, thus favored its detection, although it did not affect its occurrence. If the level of tryptophan was reduced so that the parent cells did not reach a

density above 1 x 10^7 per ml no visible growth occurred for prolonged periods until the mutants reached a density above 1 x 10^7 per ml (Fig. 25). Under the usual conditions of visible inspection of cultures, the initial invisible growth of the parent cells would have been recorded as "no growth" until, after a prolonged period of incubation, the visible turbidity due to the multiplication of the mutants was encountered. This initial apparent lack of growth in an environment adverse to the parent cells is a typical observation in many experiments where results were formerly interpreted as due to adaptation.

Generality of phenomena

In cases where no selective agent capable of eliminating the parent cells in favor of the mutant cells is known, the mutational origin of variants is more difficult to demonstrate. This applies, for example, to most antigenic variants. Yet even in these cases indirect evidence indicative of mutational changes has been obtained. Thus, Zelle[9] showed by repeated single cell isolations that 1 R cell occurred during 148 divisions of S cells obtained from an unstable *Salmonella typhimurium* strain. Stocker[10] demonstrated the mutational origin of phase variants of *S. typhimurium* and estimated their mutation rates with the help of a technique that permitted visible differentiation of variants by plating them in media containing antiserum.

Despite these various convincing demonstrations that a major proportion of bacterial variants owe their origin to spontaneous and undirected mutational changes within a small proportion of cells, there still exists a dwindling minority of dissenters who favor direct adaptative mechanisms as the major mechanism in bacterial variation. Most prominent among the latter group are Hinshelwood and his co-workers.[11, 12] They assume that cells are intrinsically capable of performing many unusual metabolic functions and that in a new environment there may occur an expansion of the appropriate enzymes or the development of already existing alternative metabolic routes.[11, 12] This, it is claimed, may lead to a direct adaptation of

[9] Zelle, M. R., J. Inf. Dis., *71*:131, 1942.
[10] Stocker, B. A. D., J. Hyg., *47*:398, 1949.
[11] Hinshelwood, C. N., *The Chemical Kinetics of the Bacterial Cell*, Oxford University Press, London, 1947.
[12] Baskett, A. C. and Hinshelwood, C., Proc. Roy. Soc., B, *139*:58, 1951.

the majority of cells in a population to novel environmental conditions, e.g., growth in the presence of a drug. Such adaptations are supposed to persist in many instances after the removal of the inductive environment. They assume an autosynthesis of cell material and the occurrence of changes in enzyme ratios under the influence of a new type of growth medium. The lag observed prior to the detection of cells with altered properties (see p. 60) is attributed to the time required for the reorganization of the cell. Furthermore, they state that "before the new steady state [in enzyme ratios] has been attained reversion occurs readily on subculture in the original medium because there is no influence to maintain the existing unstable enzyme ratios. Once a fixed, stable, ratio has been maintained for many generations, however, the whole enzyme pattern may become structurally stabilized so that it becomes a repeating unit which will not be disturbed unless important fresh adaptive changes are imposed upon the cell."[12] In view of the type of data that have been reviewed in this chapter most investigators feel that the evidence presented by Hinshelwood's group does not suffice to support a concept of widespread occurrence of hereditary adaptive changes. Although there is no doubt that adaptive mechanisms do occur (pp. 87, 101), the resulting changes, produced under the influence of a specific environment, are usually not maintained after transfer to a different environment. The weight of available evidence, therefore, forces us to conclude that a major proportion of changes in bacterial characteristics is due to spontaneous mutations. In this connection it is pertinent to point out that the spontaneous origin of mutants, independent of a specific environment, does not necessarily imply that the reactions of such mutants are identical in the absence and presence of a specific environment. For example, streptomycin-resistant mutants may display different metabolic reactions depending on whether they are growing in the absence or in the presence of streptomycin. Those reactions which they are capable of performing in the presence of streptomycin can be held responsible for their ability to grow under these normally inhibitory conditions. A mutation, therefore, may merely endow a cell and its progeny with the capacity to perform certain functions, or to display a certain phenotype, under appropriate environmental conditions.

MUTATION RATES

The conclusion that a major proportion of changes in bacterial characteristics is due to mutations is further supported by the relative stability of the altered types as expressed by their rate of mutation. The spontaneous mutation rate, usually expressed as the rate per bacterium per division, is in most cases extremely stable for any given characteristic. This can be more precisely expressed by stating that the mutation rate or stability of each gene is fairly constant. However, it must be recognized that phenotypically similar changes can be caused by mutations affecting any one of several genes. Under such conditions the mutation rate can be dependent upon the gene involved, and different mutation rates may be observed for similar phenotypic changes. Examples of commonly encountered mutation rates are presented in Table 3.

Methods for estimating mutation rates

Wherever a destructive agent capable of eliminating the parent cells in favor of the mutants is known, it is fairly simple to obtain an estimate of the mutation rate with the help of the fluctuation test (see Fig. 22). Without going into the details of the statistical considerations which underlie the calculations of mutation rates the general principle used can be stated quite simply.[13] From a series of parallel cultures, started with a small number of cells, the number of cultures containing no mutants and the number of cultures containing mutants are determined following exposure to the selective agent. This is usually accomplished by pouring the contents of each of a series of parallel broth cultures into a plate with nutrient agar containing the selective agent (phage, streptomycin, etc.) and recording the number of plates in which colonies arise following incubation. The actual number of mutants in each culture, i.e., the number of colonies developing on a plate, can be neglected in this method, thus eliminating errors that may arise because of differences in the growth rates of the mutants which may have affected their rate of establishment in the parent population (selective effects). The sole criterion used for the calculation of the mutation rate is whether or not a mutation has *occurred* in a culture. Determination of the average number of cells per culture at time of screening (exposure to phage, etc.) and knowledge of the per-

[13] See Witkin, E. M., *Methods in Medical Research*, 3:23, 1950, for more details.

TABLE 3. *Examples of mutation rates. All estimates, except those indicated by (*), are based on the fluctuation test.*

VARIATION INVOLVED	SPECIES	MUTATION RATE PER BACTERIUM PER GENERATION	REFERENCES
R ⟶ S (colonial morphology)*	*Salmonella aertrycke*	5×10^{-2}	Cold Spring Harbor Symposium, *11*:228, 1946
Flagellar antigenic phase;			J. Hyg., *47*:398, 1949
"group ⟶ specific*"	*Salmonella typhimurium*	3×10^{-4}	
"specific ⟶ group"*	*Salmonella typhimurium*	1×10^{-5}	
Pigmentation*	*Serratia marcescens*	1×10^{-4}	Cold Spring Harbor Symposium, *11*:25, 1946
Radiation resistance	*E. coli*	1×10^{-5}	Genetics, *32*:221, 1947
Threonine resistance	*Salmonella typhimurium*	4×10^{-6}	J. Bact., *62*:639, 1951
Resistance to isoniazid	*M. ranae*	3×10^{-6}	Amer. Rev. of Tuberc., *65*:768, 1952
Colonial morphology*	*Phytomonas stewartii*	1×10^{-6}	J. Agr. Res., *60*:217, 1940
Penicillin resistance	*Staph. aureus*	1×10^{-7}	Ann. Miss. Bot. Gard., *32*:131, 1945
Resistance to phage T_3	*E. coli*	1×10^{-7}	Genetics, *30*:119, 1945
Resistance to phage T_1	*E. coli*	3×10^{-8}	Genetics, *28*:491, 1943; *30*:119, 1945
Tryptophan independence	*Salmonella typhimurium*	5×10^{-8}	J. Bact., *56*:374, 1948
Histidine independence (h^- to h^+)	*E. coli*	3×10^{-8}	Genetics, *34*:72, 1949
Histidine dependence (h^+ to h^-)	*E. coli*	1×10^{-6}	Genetics, *36*:460, 1951
Utilization of itaconate as C source	*Pseudomonas fluorescens*	2×10^{-8}	J. Bact., *58*:171, 1949
Sulfathiazole resistance	*Staph. aureus*	1×10^{-9}	Genetics, *32*:249, 1947
Streptomycin dependence	*E. coli*	1×10^{-10}	J. Bact., *57*:565, 1949
Streptomycin resistance (1000 μg)	*Ps. aeruginosa*	4×10^{-10}	Pediatrics, *4*:214, 1949
Streptomycin resistance (1000 μg)	*E. coli*	1×10^{-10}	J. Bact., *57*:565, 1949
Streptomycin resistance (1000 μg)	*Shigellae*	3×10^{-10}	Pediatrics, *4*:214, 1949
Streptomycin resistance (1000 μg)	*H. pertussis*	1×10^{-10}	Pediatrics, *4*:461, 1949
Streptomycin resistance (1000 μg)	*Salmonella typhosa*	1×10^{-10}	Pediatrics, *4*:214, 1949
Streptomycin resistance (25 μg)	*Salmonella typhosa*	5×10^{-6}	Pediatrics, *5*:78, 1950
Streptomycin resistance (25 μg)	*H. pertussis*	6×10^{-10}	Pediatrics, *5*:78, 1950

centage of cultures containing no mutants suffice to estimate the mutation rate, a, per bacterium per division cycle, according to Luria and Delbrück's formula[1, 14]:

$$a = -(ln\ 2)\ (ln\ p)/N$$

where p is the fraction of cultures containing no mutants and N is the average number of bacteria per culture at the end of growth.

In the method just described, events following the appearance of mutants are disregarded, since no attention is paid to the number of mutants that established themselves in each of the parallel cultures. However, a method of estimating mutation rates by noting the average number of mutants per culture in a series of parallel cultures has also been developed,[1, 14] but it can be applied only if the growth rates of the mutant and parent strains are the same in the medium used. In this case the mutation rate is calculated according to the formula:

$$r = (aN/ln\ 2)\ ln\ (CaN/ln\ 2)$$

where a is the mutation rate bacterium division cycle, r is the average number of mutants sample, N is the average number of bacteria sample at the end of growth, and C is the number of cultures.

Other methods are based on the number of mutant clones appearing during growth on solid medium or on the highest number of mutants occurring in any one of a series of cultures.[13] More recently, improved formulae for the calculation of mutation rates, which take into consideration the delay in phenotypic expression of mutations[14] (see p. 75) and several features of population dynamics,[15] have been evolved. However, these formulae involve too many statistical considerations to be discussed here.[16]

In cases where the parent population cannot be eliminated by selective agents it is possible to estimate the mutation rate in initially homogeneous cultures on the basis of the proportion of mutant cells present after a known number of generations have elapsed, *provided the growth rates of parent and mutant cells are identical.*[10] In the latter case the proportion of mutant cells in a culture, initially

[14] Newcombe, H. B., Genetics, *33*:447, 1948.

[15] Lea, D. E. and Coulson, C. A., J. of Genetics, *49*:264, 1949.

[16] The interested reader is referred to the original publications, and Lederberg's chapter in *Bacterial Physiology*, edited by Werkman and Wilson, Academic Press, Inc., New York, 1951.

consisting of one type only and maintained in continuous logarithmic growth, will increase linearly with the number of generations at a rate proportional to the mutation rate. Under these conditions, the mutation rate $= \frac{\text{proportion of mutant cells}}{\text{number of generations}}$. Stocker used this method in a study of phase variation of *Salmonella typhimurium* (p. 117).

Mutation rates that have been determined by any of the available methods can be considered as only approximately correct. Several factors contribute to the difficulty of getting entirely accurate rates; among them are: (1) The delayed phenotypic expression of many mutational changes[14] (p. 74); (2) the existence of inhibitory effects produced by the parent cells which, in some cases, interfere with the growth of certain mutant cells (e.g., p. 163); and (3) selective effects causing differences in the growth of mutant and parent cells. The last mentioned factor plays no role in the method based upon absence or presence of mutants in a culture (p. 62), thus making it generally the method of choice.

Another factor that must be considered in tests designed to estimate mutation rates is the limited growth of the parent cells that may occur in some selective screening media supposed to support only the growth of mutant cells (p. 50). This, in turn, may lead to the occurrence of additional mutations during the period of *residual growth* of parent cells, and may introduce an error in fluctuation tests when calculations are based upon the number of cells plated instead of the final residual growth of the parent cells. For example, plating of a population of streptomycin-dependent cells on a medium free of streptomycin not only will result in the growth of a few non-dependent mutants that might be present at time of plating, but will permit the dependent cells to divide a few times (residual growth), thus increasing the chances for the occurrence of additional mutations toward non-dependence. Bertani[17] has found that the extent of such residual growth is mainly a function of the concentration of streptomycin in which the dependent cells have previously been cultured. In addition, he has shown that it is actually possible to utilize this phenomenon for a simple estimation of mutation rates from dependence to non-dependence. It is merely necessary to determine the number of colonies that develop from a streptomycin-dependent population during a known number

[17] Bertani, G., Genetics, *36*:598, 1951.

of residual cell divisions on streptomycin-free agar. Each colony can be assumed to correspond to one non-dependent mutant which either arose during residual growth or was present in the original inoculum. Apparently, the number of mutants that may be carried over in the inoculum is usually very small in comparison with the number that will arise during residual growth.[17] The approximate mutation rate, therefore, can be obtained by expressing the number of mutants present in the final population in terms of the known number of residual cell divisions. This simple method appears applicable to any system involving changes from dependence to non-dependence upon a certain growth factor. It has proved particularly useful in the analysis of certain environmental agents that are capable of enhancing the spontaneous mutation rate (p. 73).

Mutation rate vs. mutant frequency

All present methods for the estimation of mutation rates have one important feature in common. This is the recognition of appropriate adjustments necessitated by the fact that mutants present at the time of screening in any bacterial population may represent mutants which arose just prior to screening as well as the progeny of mutants which arose several generations earlier. Thus, mere inspection of cultures, even following growth from a single cell, cannot reveal the mutation rate. Such observations may indicate the frequency of certain mutants in a population *(mutant frequency)* but this does not necessarily reflect the rate at which such mutants arose *(mutation rate)*.[18] It is also incorrect to conclude that differences in mutation rates exist in cases where a comparison between cultures started from similar inocula and grown under different

[18] Only under special conditions can the mutant frequency reflect the mutation rate. For example, when a phage-susceptible population is plated on agar and phage is applied to different plates at different times after plating, the number of phage-resistant colonies that will develop after a known number of divisions of the parent cells will reflect the mutation rate. Under such conditions each mutant arising during the cell divisions that take place from the time of plating to the time of exposure to phage will give rise to one colony. The progeny of each of these mutants will remain within their respective colonies and hence every resistant colony corresponds to one mutational event. The last does not apply necessarily to the resistant cells that may have been present in the inoculum, but their number can be ascertained and subtracted by scoring the number of colonies that develop on plates exposed to phage immediately after inoculation.

physiological conditions reveals that the second culture contains a significantly greater proportion of mutants than the first one. In such cases the altered physiologic conditions may have affected only the rate of establishment of the mutants arising at identical rates under either condition. We shall discuss such considerations again in Chapter 8.

Mutation rate and cell division

As stated above, it has been customary to express mutation rates as the rate per bacterium per division, and up to recently it was believed that cell division was necessary for the occurrence of mutations. It was generally assumed that a mutation may involve a reproductive error occurring in the act of gene reproduction. Yet, in recent studies with the *chemostat,* a device which permits continuous growth of populations with constant density, Novick and Szilard[19] uncovered at least one case in which the rate of mutation was dependent on unit time rather than generation time. This means that the mutation rate was the same within a given time regardless of the number of division cycles that had occurred, an observation which is difficult to reconcile with the copy-error concept. The system under investigation was mutation from tryptophan-dependence to tryptophan-independence in *E. coli.* In addition, it has been reported that mutations affecting the pigmentation of *S. marcescens* can occur in resting cells.[20] At the present moment, it cannot be predicted how generalized this phenomenon may be, but its demonstration indicates the possibility of an increase in genetic instability even in cultures that are maintained without cell multiplication.

The cause of spontaneous mutations

Hardly anything is known about the cause of spontaneous mutations *(mutagenesis).* Many speculations have been advanced. Most prominent among them have been the possible role of cosmic rays and slight temperature fluctuations affecting the energy equilibria of the determinants of heredity characteristics. However, no proof for such speculations has as yet been obtained and the search for the

[19] Novick, A. and Szilard, L., Proc. Nat. Acad. Sci., *36:*708, 1950; Cold Spring Harbor Symp. Quant. Biol., *16:*337, 1951.

[20] Kaplan, R. W., Z. Naturforschg., *2b:*308, 1947.

causes of spontaneous genetic instability remains one of the most challenging problems of genetics. In the search for answers to this problem much attention has been given to those specific environmental agents which are capable of increasing spontaneous mutation rates. These agents are called *mutagenic agents* and they will be discussed in the following chapter.

CHAPTER 5

Mutagenic Agents and Mutagenesis

AGENTS CAPABLE OF INCREASING MUTATION RATES ARE CALLED *mutagenic agents* or *mutagens*. Mutations resulting from treatment with such agents are called *induced mutations* in contrast to the normally occurring *spontaneous mutations*. Mutagenic agents can increase mutation rates as much as 10–100,000 fold. The most widely investigated mutagens are x-rays,[1] ultraviolet rays (UV)[1] and nitrogen mustard.[2] However, many other mutagens have been discovered in work with microorganisms; among them are organic peroxides and broth treated with hydrogen peroxide,[3] carcinogenic substances,[4] acriflavine,[5] some purine derivatives,[6] certain simple chemical compounds[7] like $MnCl_2$, and even visible light if bacteria are sensitized with erythrosin prior to exposure (photodynamic effects[8]). Early investigations suggested that none of these mutagenic agents is capable of inducing specific mutations; it was believed that they merely increased the rate of undirected mutations. Some recent results,[9] however, indicate that there may be a difference between spontaneous and induced mutants. Among a large group of spontaneously occurring phage-resistant mutants all independently isolated mutants with added growth requirements were tryptophan-dependent, whereas several phage-resistant mutants isolated

[1] Demerec, M. and Latarjet, R., Cold Spring Harbor Symp. Quant. Biol., *11:* 38, 1946.
[2] Bryson, V., J. Bact., *56:*423, 1948.
[3] Wyss, O., Clark, J. B., Haas, F. and Stone, W. S., J. Bact., *56:*51, 1948.
[4] Demerec, M., Hereditas, *Supp. Vol.:* 201, 1949.
[5] Witkin, E. M., Cold Spring Harbor Symp. Quant. Biol., *12:*256, 1947.
[6] Novick, A. and Szilard, L., Cold Spring Harbor Symp. Quant. Biol., *16:*337, 1951.
[7] Demerec, M., Bertani, G. and Flint, J., Amer. Nat., *85:*119, 1951.
[8] Kaplan, R. W., Naturwiss., *35:*127, 1948.
[9] Bryson, V. and Davidson, H., Proc. Nat. Acad. Sci., *37:*784, 1951.

following UV irradiation showed completely different requirements (Table 4).

TABLE 4. *Phage resistance pattern and nutritional requirements of spontaneous and ultraviolet-induced phage-resistant mutants of* E. coli *isolated from strain B r. B r 1 refers to mutants resistant to phage T_1 only, B r 1,5 refers to mutants simultaneously resistant to phage T_1 and T_5, etc.*

(From Bryson, V. and Davidson, H., Proc. Nat. Acad. Sci., *37*:784, 1951)

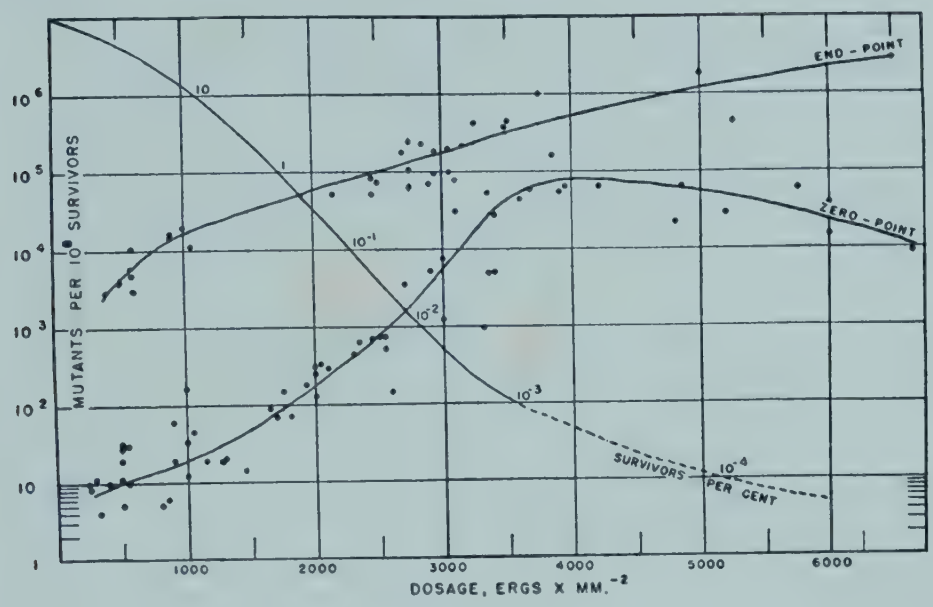
	SPONTANEOUS				INDUCED			
	Total	Non-deficient	Deficient		Total	Non-deficient	Deficient	
			Trypto-phanless	Misc.			Trypto-phanless	Misc.
B/r/1	72	20	52	0	9	7	1	1[a]
B/r/1,5	87	79	8	0	105	100	1	4[b]
B/r/1,3,4,7	4	0	4	0	0	0	0	0
B/r/1,3,4,7,6	1	0	1	0	0	0	0	0
Total	164	99	65	0	114	107	2	5

[a] methionineless

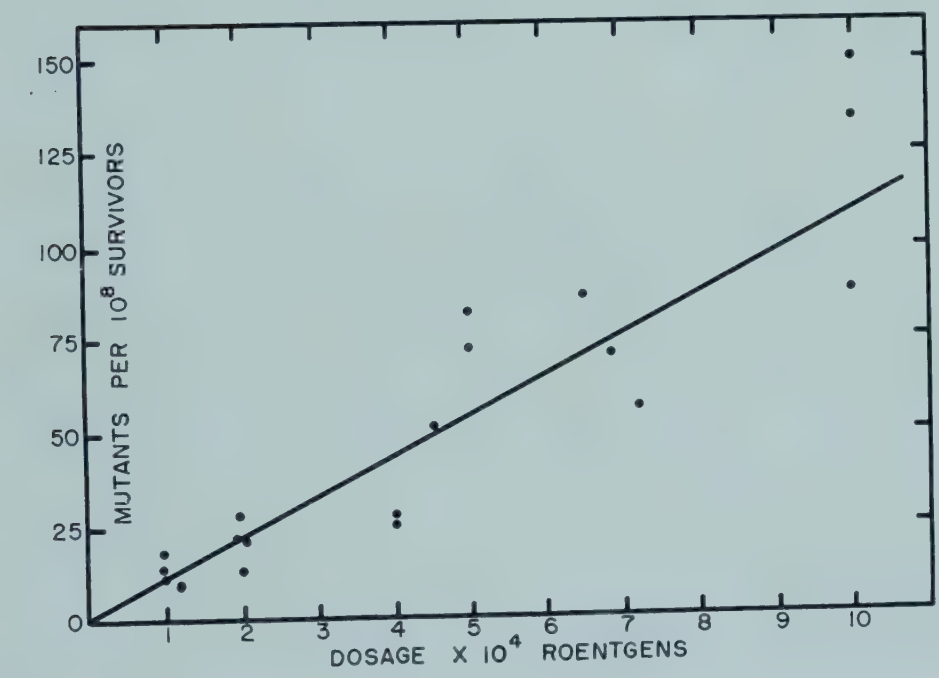
[b] thiamineless, aromaticless, purineless and serineless.

Dosage effects

It has been claimed that in the case of x-rays there is a direct relationship between dosage and mutagenic effect[1] (Fig. 26). This straight-line relationship, which had been observed earlier in studies with higher organisms, has given rise to the "single hit" interpretation of x-ray effects. This interpretation assumes that a single hit or ionization of a sensitive target, presumably the gene, causes permanent alterations in the hereditary determinant and consequently the characteristics that it controls. However, some recent observations on agents which are capable of counteracting mutagenic x-ray effects (see below) have made such a simple interpretation less likely. In the case of ultraviolet irradiation linear dose-effect relationships usually have not been observed. In most experiments a rapid initial increase of mutagenic effects with increasing UV dosage was followed by an optimum level where further increase in dosage failed to show a corresponding increase in mutation rates[7] (Fig. 26). Linear dose-effect relationships have been reported for photodynamic effects.[8]



A



B

Fig. 26. Dosage dependence curves for UV- and x-ray-induced phage-resistant mutants of *E. coli*, strain B/r. *A*, Mutants induced by ultraviolet 2537 Å (semilogarithmic plot); *B*, mutants induced by x-rays (linear plot). (After Demerec and Latarjet.[1])

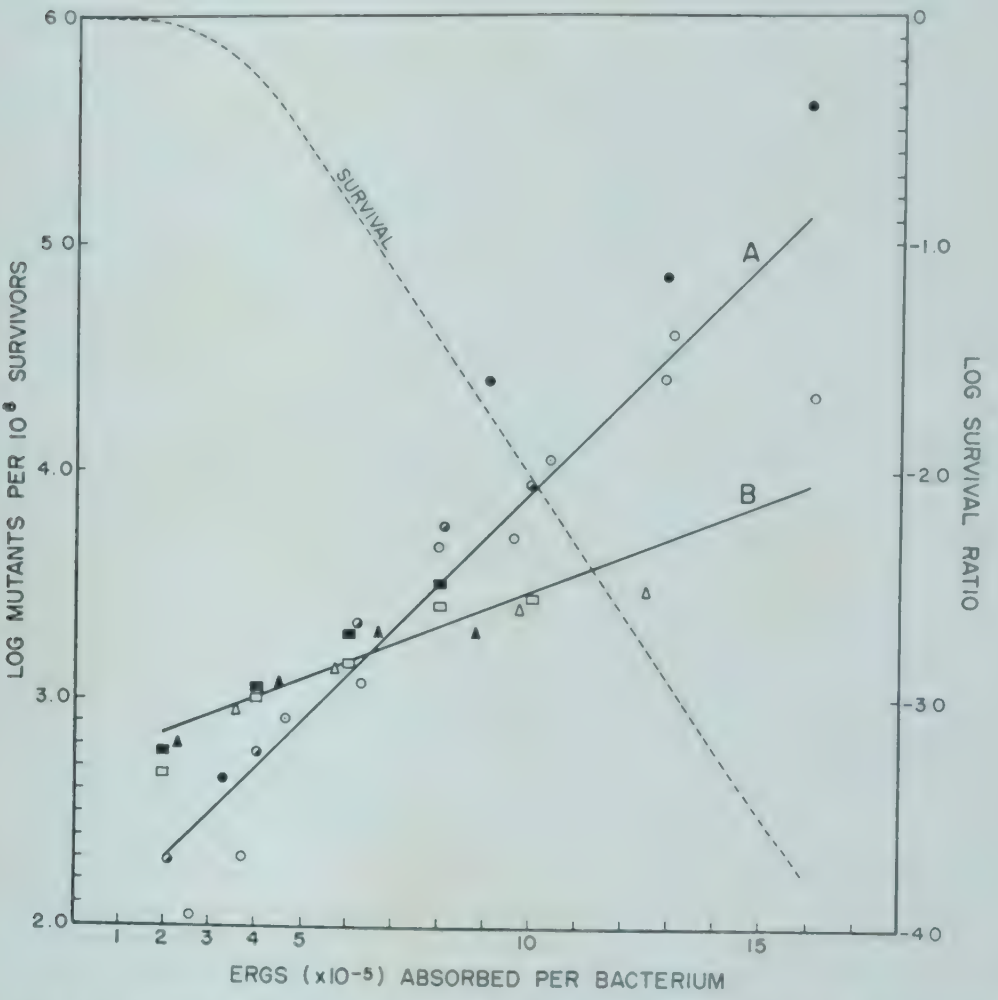


Fig. 27. Dosage dependence curves for two different types of UV-induced mutants of *E. coli*, B/r. Circles indicate purine-independent mutants obtained after exposure of purine-requiring cells to 2650 A, squares and triangles indicate streptomycin-independent mutants isolated after similar treatment of streptomycin-dependent cells. Data obtained from independent tests are indicated by differences in shading of circles, squares and triangles. Line *A* shows the least square regression of log. mutants on dosages fitted to cases shown by circles (reversion of purine requirement); line *B* shows the regression fitted to cases shown by squares and triangles (reversion of streptomycin dependence). Statistical analysis indicates that the difference in slope between lines *A* and *B* is highly significant. For purposes of comparison all data have been plotted as the log of the mutants versus ergs, even though additional data indicate that in the case of streptomycin dependence a simple linear relation between the number of mutants and dose describes the data more adequately than an exponential curve. (Based on unpublished data by M. Zelle and A. Hollaender.)

More recent observations indicate that prior generalizations in regard to typical dose-effect relationships, as illustrated in Fig. 26, may not be entirely accurate. It appears that such relationships are greatly dependent upon the type of mutation used as indicator in these studies[10] (Fig. 27). In other words, each gene may have its own characteristic response to the effects of different doses of a given mutagen.

Lethal effects and mutation rates

The majority of mutagens cause both killing as well as mutagenic effects. Thus the larger the proportion of cells that fail to survive, the greater is the increase in mutation rates. In assaying mutagenic agents it is, therefore, of great importance that the mutation rate be determined as rate among the survivors rather than as rate for the total population exposed. In older studies, certain environmental agents were believed to have increased mutation rates whereas they had merely caused a differential survival of mutants, killing a larger proportion of parent cells than of mutant cells.

Determination of induced mutation rates

Increased mutation rates following exposure to mutagenic agents can be determined with the help of the fluctuation test applied to the survivors or their progeny. In a number of studies with *E. coli*[7, 11] mutagenic effects have been tested with the help of Bertani's technique (p. 65): Streptomycin-dependent cells are plated on streptomycin-free media following treatment with a suspected mutagen; the mutation rate is then calculated from the number of non-dependent mutants that develop into large colonies on the antibiotic-free medium during a known number of residual cell divisions of the dependent cells.

Phenomic lag

The foregoing tests have revealed that following treatment with mutagenic agents a major proportion of induced mutants will not manifest themselves until several generations after exposure. Demerec,[12] employing the phage technique (see footnote on p.

[10] Unpublished data by M. Zelle and A. Hollaender, and by H. Plough.

[11] Demerec, M. and Hanson, J., Cold Spring Harbor Symp. Quant. Biol., *16*:215, 1951.

[12] Demerec, M., Proc. Nat. Acad. Sci., *32*:36, 1946.

TABLE 5. *Delayed appearance of phage-resistant (B 1) mutants after ultraviolet treatment of* E. coli, *strain B. Cells were exposed to ultraviolet irradiation for 2 or 4 minutes and were subsequently incubated for various lengths of time before application of phage.*

(After Demerec[12])

INCUBATED BEFORE PHAGING	PERIOD OF EXPOSURE TO UV						CONTROL		
	4 minutes			2 minutes					
	Total no. of bacteria	B/1	Mutant frequency x 10^8	Total no. of bacteria	B/1	Mutant frequency x 10^8	Total no. of bacteria	B/1	Mutant frequency x 10^8
0	1.12 x 10^7	19	170	1.23 x 10^7	6	49	2.42 x 10^7	0	
2 hrs	1.79 x 10^7	118		2.71 x 10^7	95		2.4 x 10^8	4	
Increment 0–2 hrs	0.67 x 10^7	99	1478	1.48 x 10^7	89	601	2.2 x 10^8	4	1.8
3 hrs	1.43 x 10^8	548		2.17 x 10^8	414		19.2 x 10^8	55	
Increment 2–3 hrs	1.25 x 10^8	430	344	1.90 x 10^8	319	168	16.8 x 10^8	51	3.0
4 hrs	11.44 x 10^8	2081		17.36 x 10^8	1234		153.6 x 10^8	295	
Increment 3–4 hrs	10.01 x 10^8	1533	153	15.19 x 10^8	820	54	134.4 x 10^8	240	1.8

66), was the first to observe that after exposure to x-rays the percentage of recoverable mutants increased during successive generations after treatment (Table 5). The frequency returned to normal approximately 8 generations after treatment. Similar delayed effects, for which Davis coined the term *phenomic lag*, have been observed after treatment with almost all mutagens; Newcombe[13] has demonstrated that a similar phenomic lag also occurs in spontaneous mutations. This means that mutations frequently will not express themselves phenotypically until some time after their occurrence. Several causes have been suggested for this delayed phenotypic expression: (1) time required for the exhaustion of preformed, stored products which continue to be utilized but can no longer be synthesized as a result of the mutation (physiologic lag); (2) involvement of cytoplasmic factors; (3) delayed growth of cells affected by mutagens; and (4) segregation of the hereditary determinants, especially segregation of affected nuclei from multinucleate cells. The last phenomenon can play an important role but by itself cannot explain all observations made on phenomic lag. It deserves a brief description here because it illustrates a phenomenon that can occur with multinucleate cells, a subject which has hitherto been insufficiently investigated and must be considered in genetic studies with bacteria which are known frequently to contain more than one nucleus. Figure 8 illustrates the effect which may occur when a mutation takes place in one nucleus of a multinucleate cell. The likelihood of a spontaneous or induced change occurring in more than one nucleus at the same time is practically nil. Since mutation rates are usually in the neighborhood of 1×10^{-7}, two simultaneous changes would have a probability of $1 \times 10^{-7} \times 1 \times 10^{-7}$, or 1×10^{-14}, a slim probability indeed. Thus when one nucleus in a multinucleate cell changes, the change, if recessive, would not express itself phenotypically in the presence of the normal, unchanged genes or alleles in the other nuclei. Most mutational changes are recessive and, therefore, cannot express themselves phenotypically until the changed nucleus finds itself within a cell without other nuclei or in the presence of similar changed nuclei (Fig. 8). This process requires cell divisions and therefore can account for some of the delays observed in phenotypic expressions of mutations.

[13] Newcombe, H. B., Genetics, *33*:447, 1948.

Nuclear structures as sites of mutational changes

Experiments which have taken such nuclear segregations into consideration have also assisted in demonstrating that nuclear structures actually are the sites of mutational changes in bacteria. Witkin[14] has irradiated cells of *E. coli* when they were predominantly either uninucleate or multinucleate. Such cells can be obtained at different stages of a culture's growth and the number of nuclei per cell can be checked by direct cytological observations. Witkin used

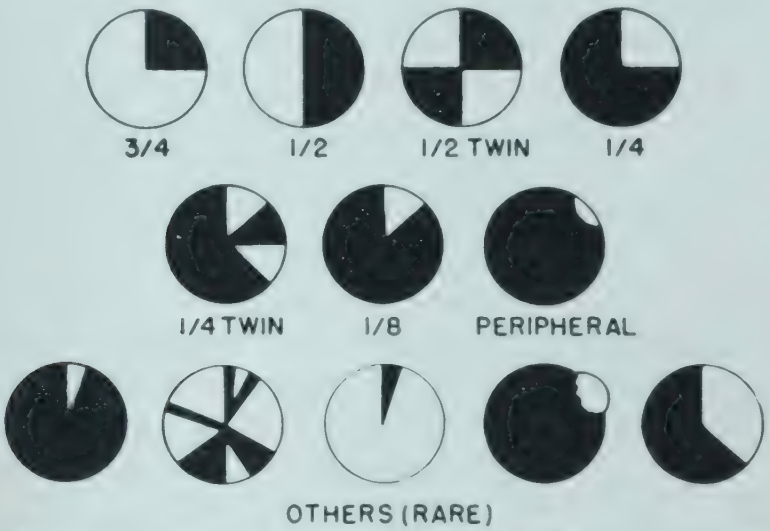


Fig. 28. Diagrammatic representations of type of lactose-negative sectored colonies obtained on EMB agar after treatment with ultraviolet light. (E. Witkin.)

mutations involving inability to ferment lactose (lac$^-$) as indicators; they can be easily recognized by the white appearance of colonies developing from lac$^-$ cells on eosin-methylene blue (EMB) agar; the lac$^+$ parent cells give rise to red colonies on EMB agar. When a population of primarily uninucleate lac$^+$ cells was irradiated and plated on EMB agar, a few white colonies developed, i.e., colonies which were entirely lac$^-$. Obviously mutations to lac$^-$ which occurred in a few cells expressed themselves immediately, thus leading to the development of colonies which consisted entirely of lac$^-$ cells. However, when lac$^+$ populations consisting primarily of cells with two nuclei were irradiated, the development of several sectored colonies, half lac$^-$ and half lac$^+$, was observed (Fig. 28). In this case the mutation occurred in only one of the two

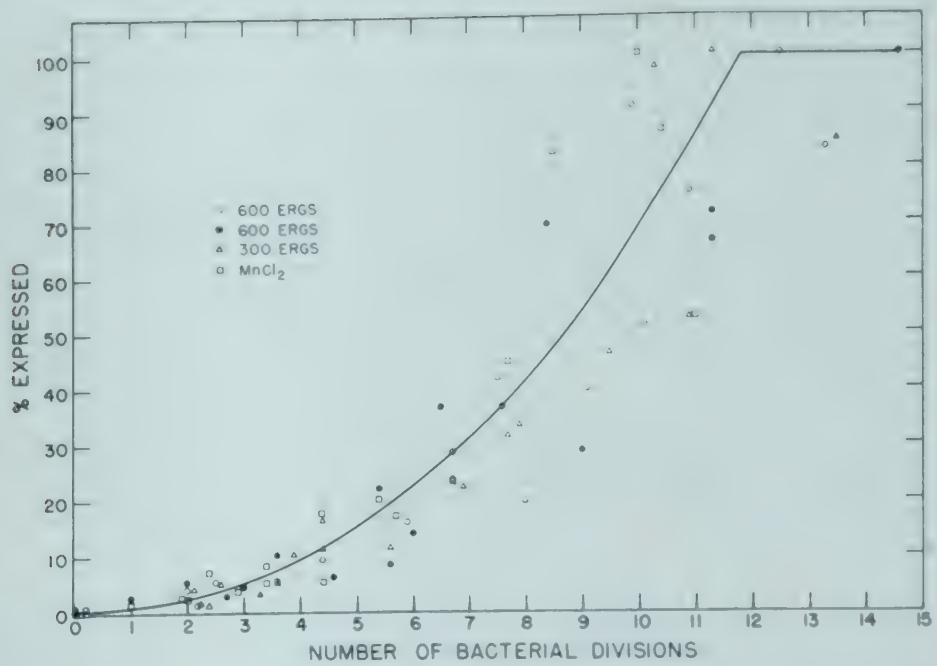
[14] Witkin, E. M., Cold Spring Harbor Symp. Quant. Biol., *16*:357, 1951.

nuclei of the cell. Since half of the subsequent colonial growth originated from the mutated nucleus and the other half from the unaffected nucleus, only half of each colony represented the progeny of the nucleus in which the mutation occurred. Similarly, irradiation at stages where most cells contained four nuclei resulted in the development of a certain proportion of colonies showing quarter sectors of lac$^-$ cells (Fig. 28). Although these results indicate that nuclear phenomena and mutational phenomena show a high degree of association, this situation is not quite as simple as first believed. Occasional sectoring in colonies, indicative of delayed mutational changes, was observed in later studies even when uninucleate cells were exposed to mutagenic agents.

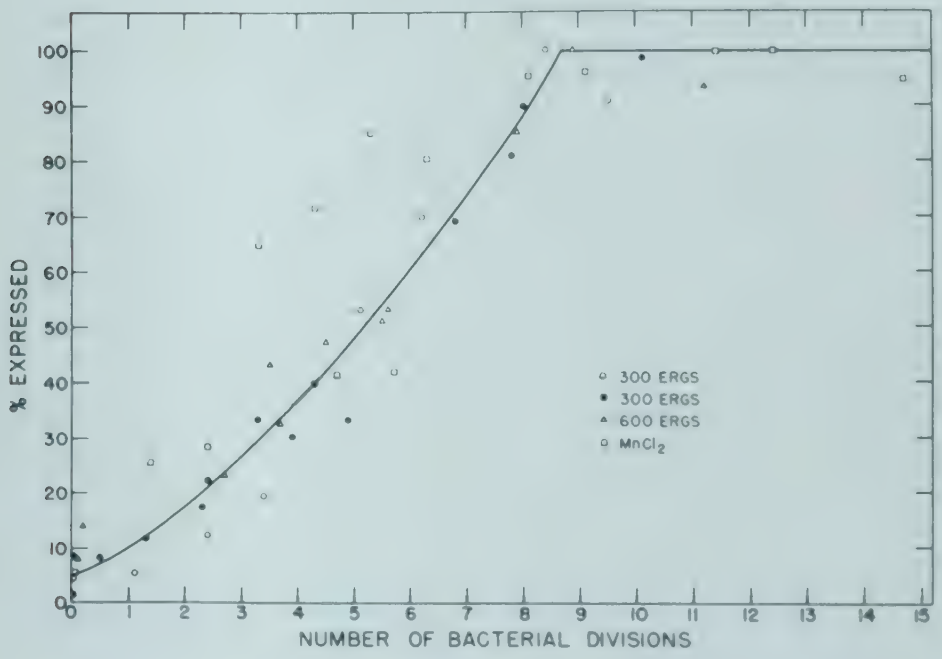
Mutational lag

Additional evidence, indicating that the mutational events may be delayed for some time following the actual exposure to mutagenic agents, is accumulating. Demerec and Cahn[15] made a detailed study of the delayed appearance of mutants in three strains of *E. coli* with specific growth-factor requirements in which a relatively high rate of reversions to lack of such requirements could be induced with help of different mutagens. They found that each kind of mutant had its own characteristic pattern of delayed appearance. Figure 29 shows some of these results and also illustrates that in each case the pattern of delayed appearance is not affected by the use of different mutagens or different intensities of radiation dosage. Each genetic locus appears to have its own characteristic pattern of behavior which is independent of the mutagen initiating the reaction. Since, even in cultures started from similar inocula, the delayed appearance is not similar for all mutants, Demerec and Cahn have concluded that mutational lag rather than phenomic lag was responsible for their results. In view of such findings it can be postulated that the delayed occurrence of mutants may be caused by mutational lag as well as by phenomic lag. The existence of mutational lag further indicates that the effects of mutagenic agents are not direct but involve a series of intermediate steps. Once affected, the gene may remain in an unstable state for some time before either reverting to its original condition or mutating.[15, 16, 17]

[15] Demerec, M. and Cahn, E., J. Bact., *65*:27, 1953.
[16] Auerbach, C., Cold Spring Harbor Symp. Quant. Biol., *16*:199, 1951.
[17] McElroy, W. D., Science, *115*:623, 1952.



A



B

Fig. 29. Mutational lag. *A*. The rate of appearance of induced reverse (prototrophic) mutants of *E. coli*, strain M12-11 (methionine-requiring) during approximately 14 bacterial divisions following treatment; *B*. similar data obtained with *E. coli*, strain MT12-66 (methionine- and threonine-requiring). (From Demerec and Cahn.[15])

"Zero-point" and "delayed" mutants

Mutants which express themselves immediately following exposure to a mutagenic agent have been labelled *zero-point* mutants. In contrast, mutants which do not show the phenotypic change until one or more cell divisions following exposure to mutagenic agents, either due to phenomic lag or delayed mutation, have been called delayed mutants.[1] Recent studies have cast some doubt on the existence of zero-point mutants.

The delayed mutants are of considerable practical interest in studies which are designed to isolate an optimal number of mutants following exposure to a mutagen. In such studies irradiated or otherwise treated bacterial populations should not be exposed immediately to the screening agent but a growth period involving approximately 3 to 6 divisions should be allowed before screening (see p. 94).

Photoreactivation

When ultraviolet irradiation is used for increasing mutation rates, a phenomenon called *photoreactivation* must be considered. Photoreactivation was first described by Kelner[18] who observed that the "killing" effects of ultraviolet irradiation can be significantly diminished if bacteria are exposed to visible light following the ultraviolet irradiation treatment. The mutagenic effects of ultraviolet irradiation are similarly decreased if treated cells are exposed to visible light. With the help of photoreactivation it has been possible to demonstrate that the mutagenic effects of ultraviolet irradiation can differ from the lethal effects produced by such irradiation. After exposure to low doses of ultraviolet irradiation both the lethal and mutagenic effects can be greatly reduced by post-treatment with light, whereas with high doses the mutagenic effect becomes stable and only the lethal effect can be reversed.[19]

Other agents capable of counteracting the lethal and mutagenic effects of x-ray and ultraviolet irradiation have been discovered by Hollaender and his colleagues.[20] They found that oxygen is a critical factor; irradiation in the absence of O_2 significantly reduces both killing and mutagenic effects of irradiation. Cells grown anaerobi-

[18] Kelner, A., J. Bact., *58:*511, 1949.

[19] Newcombe, H. B. and Whitehead, H. A., J. Bact., *61:*243, 1951.

[20] Hollaender, A., Baker, W. K. and Anderson, E. H., Cold Spring Harbor Symp. Quant. Biol., *16:*315, 1951.

cally were found to be more resistant to irradiation effects than aerobically grown cells. The presence of reducing substances like BAL, cysteine, or $Na_2S_2O_4$ in the culture medium also decreased both the mutagenic and the killing effects of irradiation,[20] but the effects of some of these substances, e.g., cysteine, may be caused by a physical absorption of the mutagenic rays rather than by a true physiological protection.[21] Apart from their practical importance these observations also indicate the existence of intermediate steps in the action of radiation.

The belief is growing that all mutagenic effects involve such intermediate steps; possibly these may be in the nature of increased production of some mutagenic metabolites. The existence of intermediate steps in mutagenesis is further indicated by the more recent discovery of some true anti-mutagens, i.e., substances that are capable of reducing spontaneous mutation rates. In studies with *E. coli* it has been observed that guanosine, adenosine and inosine prevent mutations from being induced by theophylline or caffeine and in addition they reduce the spontaneous mutation rate to one half or one third.[22]

[21] Whitehead, H. A., Science, *116*:459, 1952.

[22] Novick, A. and Szilard, L., personal communication.

CHAPTER 6

Representative Types of Mutants: I

AMONG BACTERIAL MUTANTS, OBTAINED EITHER SPONTANEOUSLY or with the help of mutagenic agents, certain types have been extensively analyzed by both geneticists and bacteriologists. They are mutants with altered resistance to inhibitory agents, especially antibiotics, mutants displaying novel growth-requirements or fermentation characteristics, and mutants involving changes in colonial morphology, virulence and antigenic characteristics.

MUTANTS RESISTANT TO INHIBITORY AGENTS

Streptomycin resistance

One-step mutants exhibiting an absolute increase in resistance to certain inhibitory agents, namely streptomycin-resistant mutants and phage-resistant mutants, have already been referred to in the foregoing chapters. Their isolation is relatively simple; all that is required is exposure of large, predominantly sensitive populations to phage or streptomycin, whereby all sensitive cells are inhibited and only the small proportion of resistant mutants, e.g., 1 in 10^9, continues to multiply. Thus plating of 10^9 cells from a phage-susceptible population on media containing phage will yield several colonies, each consisting of clones originating from a phage-resistant mutant. These mutants are absolutely resistant to the phage and thus represent one-step qualitative changes in properties which are undoubtedly connected with changes in some biosynthetic step. In the case of streptomycin-resistance, mutants can be isolated in one step that are resistant to the highest attainable concentration of the antibiotic. However, when a large streptomycin-susceptible population is plated on streptomycin-containing agar, mutants resistant to the highest level as well as mutants resistant to lower levels of streptomycin will be found. Thus plating on media containing, let us say, 100 μgm of streptomycin per ml will yield mutants resistant to an optimum concentration of streptomycin, others which are

only resistant to 100 μgm ml of streptomycin, and still others which upon subsequent subculturing will be found to be streptomycin-dependent (Fig. 30). Mutants resistant to different levels of streptomycin can arise at different rates (see Table 3). Demerec[1] has proposed that this occurrence of mutants with different levels of streptomycin-resistance involves mutations of genes with unequal potency, which means that in one cell a gene may mutate and provide maximum resistance, whereas in another cell a different gene

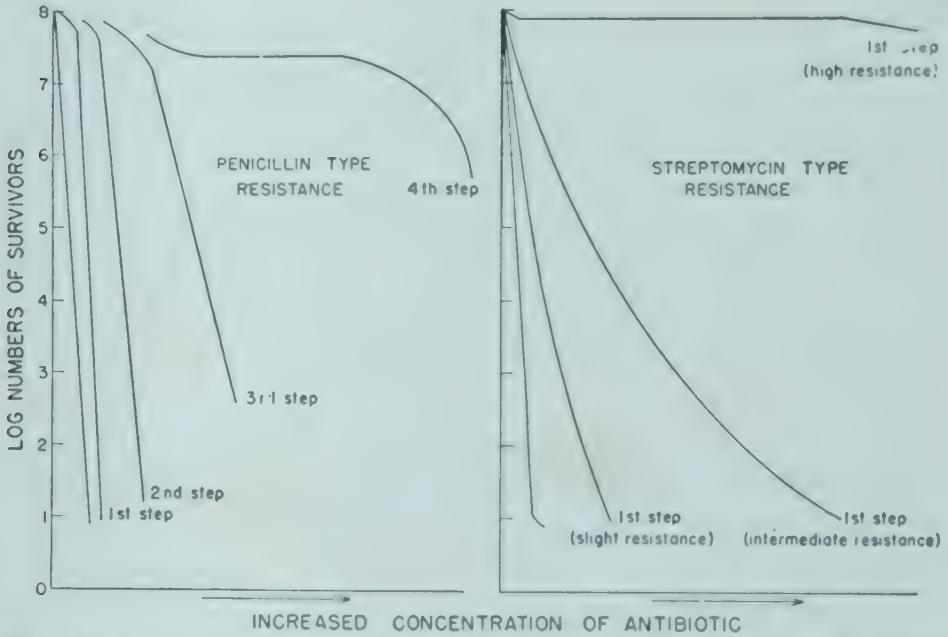


Fig. 30. Comparison of survival curves, showing the penicillin and streptomycin types of resistance pattern. (From Bryson and Demerec, Ann. N. Y. Acad. Sci., *53*:283, 1950.)

may mutate and thereby afford only intermediate resistance. This hypothesis is helpful in contrasting the possibility of obtaining in one step mutants which display low, intermediate, or high streptomycin resistance with the impossibility of obtaining in one step highly resistant mutants to any of the various other antibiotics, except isonicotinic acid hydrazide.[2]

Penicillin resistance

Let us take penicillin-resistance as an example which applies equally to resistance to most other antibiotics, e.g., aureomycin,

[1] Demerec, M., J. Bact., *56*:63, 1948.

[2] Szybalski, W. and Bryson, V., Am. Rev. Tuberc., *65*:768, 1952.

chloramphenicol, neomycin, Terramycin, etc. When a population of penicillin-sensitive cells is inoculated into a medium containing high levels of penicillin, no resistant mutants appear. However, if such a susceptible population is cultured in the presence of low levels of penicillin, mutants resistant to these low levels can be isolated and the latter cells when cultured in the presence of slightly higher levels of penicillin will permit the subsequent isolation of even more resistant mutants (Fig. 30). Thus penicillin-resistance increases stepwise. Mutants resistant to high levels cannot be isolated in one step but require several mutational steps each of which increases the resistance level in approximately geometric order. Demerec[1] has suggested that this stepwise increase in resistance involves changes of genes with equal potency. To visualize this interpretation let us set the value of each mutational change (or the potency of the altered gene) at 2 units. A first mutational change would result in resistance to 2 units. A subsequent mutation, also of the potency 2, would raise the resistance level to 4, a further mutational change adding a third 2 potency would raise the resistance level to 8, the next change to 16, etc. Because of the low rate of each mutational step the likelihood of two of these changes occurring simultaneously is practically nil.

Isolation of antibiotic-resistant mutants

To accomplish the isolation of mutants highly resistant to antibiotics other than streptomycin, or isonicotinic acid hydrazide, it is necessary to ascertain first the highest antibiotic concentration at which survivors can be isolated. Such survivors are usually first-step mutants. Doubling of the concentration used in the isolation of first-step mutants and culturing of the latter cells in this higher concentration will lead to the isolation of second-step mutants. In this manner, by increasing the antibiotic concentrations, isolation of survivors from each level used and subsequent exposure of such survivors to higher concentrations, mutants resistant to very high concentrations can be obtained. However, this method is cumbersome and short-cuts have been devised for cases where knowledge of the actual quantity of antibiotic used in each step can be neglected. In one highly efficient technique, solid media are employed and each plate used for the isolation of mutants contains gradient concentrations of the inhibitory agent[3]. Such gradients are

[3] Szybalski, W. and Bryson, V., J. Bact., *64*:489, 1952.

produced by pouring a layer of nutrient agar into a tilted Petri dish, permitting this layer to solidify, and subsequently pouring a layer of antibiotic-containing nutrient agar on top of the first layer while the plate is standing level (Fig. 31). During subsequent incubation, the downward diffusion of the antibiotic results in its dilution proportional to the thickness ratio of agar layers, and a uniform concentration gradient is thus established. In order to isolate resistant mutants a high concentration of susceptible cells (approximately 1 x 10^{10} ml) is incorporated into the upper layer.

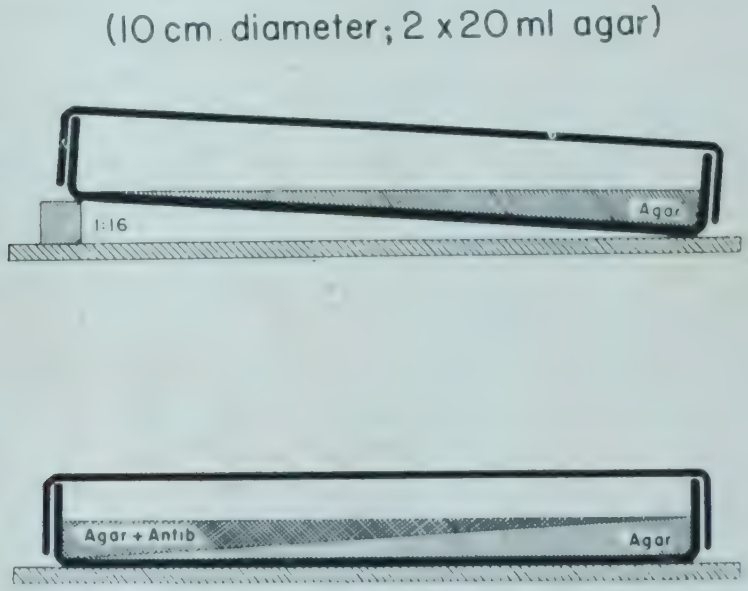


Fig. 31. Preparation of a gradient plate. (From Szybalski and Bryson.[3])

Following incubation, a dense area of growth up to the limit of the tolerated, but unknown, antibiotic concentration will be noted. Beyond this region a few isolated colonies may be found in areas of higher concentration of the inhibitory agents (Fig. 32). These colonies represent clones from resistant mutants and they now may be restreaked towards areas of higher drug concentration or they may be used for the preparation of inocula for similar layer gradient plates with a 2 to 5 times higher antibiotic concentration. A continuous culture device permitting the "automatic" isolation of highly antibiotic-resistant mutants has also been described.[4] In this "turbidostatic selector" a simple electronic circuit controls the periodic addition of nutrients to the growth-tube whenever the culture reaches a fixed turbidity, and a proportional-feed system automat-

[4] Bryson, V., Science, *116*:48, 1952.

ically, and at a geometric rate, increases the concentration of the toxic substance in the nutrient in response to the reproduction of the cells.

Mutants resistant to inhibitory chemicals, such as dyes, metal salts, etc., show a similar stepwise increase in resistance and can

Fig. 32. Growth of *E. coli*, strain B 'r, on a gradient plate containing penicillin. The high concentration of the antibiotic is on the right hand side. Resistant colonies are seen beyond the boundary delimiting the inhibitory concentration for the majority of cells. Two resistant colonies have been streaked out, resulting in the production of second step resistant colonies in the area just to the right of the end of the confluent streaks. (From Szybalski and Bryson.[3])

be isolated by repeated transfers in media containing increasing concentrations of the inhibitory agent or by the techniques just described.

Cross resistance and collateral sensitivity

The gradient plate technique described above can also be used for a rapid determination of comparative resistance by streaking a

bacterial suspension over the agar surface parallel to the axis of the concentration gradient. The length of the growing streak can

TABLE 6. *Cross resistance relationship of certain antibiotics as tested with strain B of E. coli.**

(From Szybalski, W. and Bryson, V., J. Bact., *64*:489, 1952.)

	BACTERIA			STREPTOMYCES								FUNGI
	POLYPEPTIDES											
ANTIBIOTIC → / STRAIN ↓	BACITRACIN	POLYMYXIN B	CIRCULIN	VIOMYCIN	STREPTOTHRICIN	NEOMYCIN	CATENULIN	STREPTOMYCIN	NETROPSIN	AUREOMYCIN	TERRAMYCIN	CHLOROMYCETIN	PENICILLIN
B	1	1	1	1	1	1	1	1	1	1	1	1	1
B/r	1	1	1	1	1	1	1	1	1	1.5	1.5	1.5	1.5
/BC	[20]	1/20	1/100	1/1.5	1/1.5	1.1	1	1	1	1	1/2	1	1.5
/PB	1	[20]	10	1	4	4	3	10	2	1	1	1	1/1.5
/CR	1	4	[7]	1.1	4	4	2	4	1	1	1/1.1	1	1/1.5
/VM	1.1	1.1	1	[10]	5	4	4	5	2	1	1	1	1
/ST	1.1	1	1	20	[50]	15	7	10	1	1	1	1	1/1.1
/NM	1	1/2	1/10	2	15	[70]	15	5	1	1	1	1/1.5	1/2
/CT	1.1	1/1.5	1/1.5	2	15	70	[30]	5	1	1	1	1	1
/SM	2	1/2	1/2	1	3	2	5	[>1000]	1	1/2	1/2	1/1.5	1/2
/NT	1	1.1	1	1	1	1	1.1	1	[100]	30	10	10	60
/AU	1/1.1	1/2	1/2	1	1.1	1.1	1.1	1.1	80	[90]	30	20	30
/TM	2	1/2	1/2	1	1.1	1.1	1	1.5	80	20	[30]	5	50
/CM	2	1/40	1/50	1.1	1.1	1.1	1	2	120	50	40	[130]	80
/PN	1.1	1.1	1	1	1	1	1	1	3	1.5	2	1.5	[70]
SENSITIVITY OF B (mcg/ml)	700	20	60	50	4	2	6	2	8	1	1	1	5

* Figures in the table columns represent the factor of resistance (fold increase) compared with the parent strain set as unity (first row). Figure 1.1 designates slightly increased resistance; other figures are rough estimates. Increased sensitivity (collateral sensitivity) is shown by a fraction. The bottom row indicates the concentration of antibiotic, heading each vertical column, required to inhibit the parent strain. Framed and cross-hatched areas emphasize the high degree of cross resistance within groups of similar antibiotics. Unidirectional hatching indicates non-reciprocal cross resistance. Non-hatched frames point out a high degree of collateral sensitivity. Heavily outlined boxes on the diagonal show the resistance factor of each experimental strain to the antibiotic used in its selection. Resistant derivatives of strain B are listed in the first vertical column, in the same sequence as the antibiotics heading the table. Resistance to radiation (r) or to a specific antibiotic (abbreviation in the first column) is conventionally represented by a bar.

then be used as a direct measure of the inhibitory concentration. In this manner Szybalski and Bryson[3] have tested the degree of

cross resistance among various antibiotic-resistant mutants of *E. coli*. As shown in Table 6, bacteria resistant to one antibiotic may display highly increased resistance to another antibiotic with which they had no previous contact. The cross resistance patterns fall into several distinct groups, as indicated by shading in Table 6. However, it appears that the patterns uncovered in *E. coli* are not always similar in other species. The cause of such cross resistance can be ascribed to several factors: (1) the chemical similarity of the toxic agents; (2) their interference with the same metabolic pathway, in which case cells resistant through use of an alternative pathway would be immune to all agents affecting the original pathway; (3) the development of a non-specific biological change, such as decreased permeability; or (4) a change that affects a precursor reaction of several subsequent, diverse reactions (see Fig. 10). In addition, it has been observed that when a strain becomes more resistant to one antibiotic it may become considerably more sensitive to another (see fractions in Table 6). This phenomenon has been termed *collateral sensitivity* and may be regarded partly as the result of a selective process. In isolating strains resistant to one antibacterial substance, associated characteristics producing higher sensitivity to another antibiotic may be selected at the same time.

Non-mutational increases in antibiotic resistance

Although there is scarcely any doubt that the usual mechanism of development of resistance to antibiotics involves undirected spontaneous mutations, some evidence exists indicating that resistance to low levels of antibiotics may also occur by a process akin to a long-term adaptation[5,6]. Such phenomena, if verified by future studies, might involve cytoplasmic mechanisms rather than mere nuclear control of resistance characteristics, similar to the cytoplasmic control of cytochrome oxidase systems in yeast, analyzed by Ephrussi (p. 25). Unfortunately, hardly any experimental data regarding the role of cytoplasmic factors in bacterial heredity are available so far.

Antibiotic resistance in relation to therapy

Mutants resistant to antibiotics may establish themselves *in vivo*, e.g. in patients under treatment with antibiotics.[7] Due to the one-

[5] Gibson, M. I. and Gibson, F., Nature, *167*:113, 1951.

[6] Eagle, H., Fleischman, R. and Levy, M., J. Bact., *63*:623, 1952.

[7] See Garrod, L. P., Bull. of Hyg., *25*:539, 1950, for numerous references.

step increase to high resistance in the case of streptomycin, streptomycin-resistant mutants have been isolated from patients very frequently. However, mutants resistant to other antibiotics have also been isolated from patients under treatment. In fact, the natural occurrence of resistant strains has increased steadily since the advent of antibiotic therapy. To avoid the undesirable occurrence of antibiotic-resistant mutants in therapy, it has been suggested that two antibiotics be given simultaneously, especially when streptomycin is one of the drugs employed. Since the mutation rate to antibiotic resistance is relatively low and since resistance to one antibiotic can be entirely independent of resistance to other antibiotics, e.g., aureomycin-resistant mutants display no increased resistance to streptomycin, the chance of occurrence of mutants simultaneously resistant to both antibiotics is as good as nil. Also, if antibiotics other than streptomycin or isonicotinic acid hydrazide are given singly they should be employed at high levels because, as discussed above, resistance to these drugs develops by stepwise mutations, and low *in vivo* levels of the antibiotics, therefore, would favor the establishment of first-step mutants. High levels of the antibiotic *in vivo* would provide conditions under which first-step mutants are unable to survive.

Radiation resistance

Witkin[8] first demonstrated that radiation-resistant mutants can be isolated from *E. coli*, strain B. This type of mutation is similar to that encountered in phage resistance insofar as a one-step mutation is involved. However, in contrast to phage resistance this increase in resistance is not absolute but only relative. Witkin's technique for the isolation of radiation-resistant mutants is an interesting example of methods that may be applicable wherever one-step mutants leading to partial resistance are involved and such techniques as gradient plating are inapplicable. Figure 33 shows survival curves for radiation-susceptible (B) and more radiation-resistant (B r) cells of *E. coli* following exposure to increasing intensities of ultraviolet irradiation. It can be noted that the survival curves differ strikingly in the region of lower irradiation but differ less in the area of high irradiation intensities. Exposure of a strain B population to intensities which suffice to eliminate the sensitive parent cells, therefore, would also affect the survival of the few

[8] Witkin, E., Genetics, *32*:221, 1947.

B/r mutants present in a large population (1 in 10^5 cells). In order to isolate the B/r mutants effectively, Witkin devised the multiple irradiation method. A population of sensitive cells is first exposed to an intensity of ultraviolet irradiation capable of killing 50 per cent of the B cells. As Figure 33 shows, B/r cells are not affected at this level of irradiation and their proportion in relation to the sensitive parent cells, therefore, increases among the survivors. In addition, the initial division of the surviving B cells is retarded so that the proportion of B/r cells will increase further during a

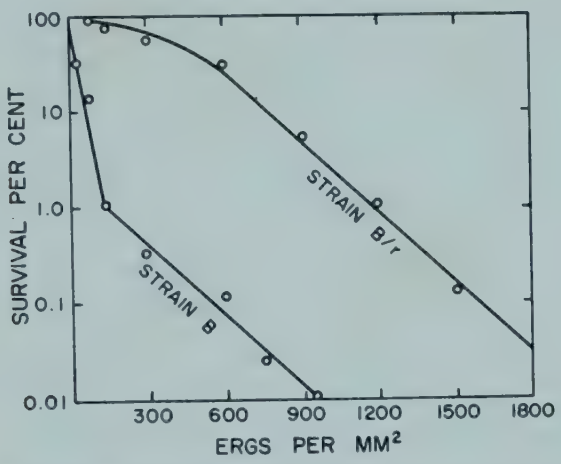


Fig. 33. Sensitivity of strains B and B/r of *E. coli* to ultraviolet irradiation. (After Witkin.[8])

subsequent growth-period of 24 hours. Samples of the 24-hour culture of survivors from the first irradiation are then exposed to another ultraviolet irradiation capable of killing 50 per cent of the B cells but none of the B/r cells. The survivors of the second irradiation are again permitted to grow for 24 hours and by now the proportion of B/r cells in the population has increased sufficiently to permit a third exposure to ultraviolet irradiation, this time to intensities which will kill all the sensitive B cells but will permit the survival of a sufficient fraction of the B/r cells. This triple exposure process can be simplified by a method which also permits the quantitative estimation of B/r mutants in a B population. A population of approximately 10^6 sensitive cells is plated on nutrient agar and exposed at an interval of 3 hours to two irradiations, each of which is capable of killing 90 per cent of B cells but practically none of the B/r mutants present. During the interval of 3 hours the B/r cells divide and form microcolonies, each con-

sisting of about 50 cells. The surviving B cells, on the other hand, fail to divide but form elongated filaments (Fig. 16). Following the second exposure the number of surviving B cells is further reduced whereas the B r cells continue their growth into microcolonies. A third ultraviolet exposure capable of killing all of the B survivors is then given. This last exposure also kills a large proportion of B r cells but approximately 100 such cells survive in each microcolony which upon subsequent incubation will develop into visible colonies consisting usually entirely of B r cells. A simple test can then be made to ascertain whether all survivors represent B r cells. This test utilizes as criterion the inhibition of division of B cells following exposure to intermediate ultraviolet intensities as shown in Figure 16. The sample to be tested is streaked on agar, exposed to ultraviolet, and inspected under a microscope after three hours of incubation. B cells will appear as long filaments, B r cells as microcolonies.

Mutants with increased ultraviolet resistance have also been found to be more resistant to peroxide, nitrogen mustard, crystal violet, safranin, potassium tellurite, and proflavine.[9] In fact, it has been demonstrated recently that radiation-resistant *E. coli* mutants can be obtained simply by isolating crystal violet- or proflavine-resistant mutants by means of the simple techniques previously described for obtaining antibiotic-resistant mutants.[10] In view of these and other data it is believed that most radiation-resistant mutants represent mutants resistant to oxidizing influences. The exact nature of this resistance is still unknown.

BIOCHEMICAL MUTANTS

Even though most mutational changes must involve in one way or another a change in biochemical processes, the term *biochemical mutants* has been used in a more restricted sense to denote mutants that have lost or gained a nutritional requirement or show changes in easily recognizable enzymatic processes, e.g., fermentation.

AUXOTROPHIC MUTANTS

The most widely investigated group of biochemical mutants includes the so-called *auxotrophic mutants*, an auxotroph being a type which requires growth factors not needed by the parent cells, or

[9] Bryson, V., Proc. Nat. Acad. Sci., *37*:784, 1951.
[10] Bryson, V., personal communication.

prototrophs. For example, prototrophic or wild-type cells of *E. coli* are able to grow in a *minimal medium* of the following composition[11]:

	grams/liter
Glucose	1
KH_2PO_4	2
K_2HPO_4	7
Na citrate · $5H_2O$	0.5
$MgSO_4 \cdot 7H_2O$	0.1
$(NH_4)_2SO_4$	1

A mutant requiring leucine or thiamine, for example, cannot grow in this minimal medium but will grow if the medium is enriched with the specific requirement or if a *complete medium* is used. A complete medium can be prepared from commercially available nutrient broth or agar or can be made up as follows[11]:

	grams/liter
Casein digest (NZ case)	10
Yeast extract	5
KH_2PO_4	1
K_2HPO_4	3
Glucose	5

Isolation procedures

It is relatively simple to isolate a prototrophic mutant from a population of auxotrophs by plating a sufficiently large population on minimal agar. The parent cells will be unable to grow, whereas any prototrophic mutant present, usually 1 in 10^8 cells, will develop into a visible colony. However, in order to isolate auxotrophs from prototrophic populations more complicated methods must be employed.

The oldest, simplest but least efficient method is to isolate at random a large number of colonies from an irradiated culture plated on complete medium. Each colony is subcultured on complete medium as well as on minimal medium. If growth fails on minimal medium but occurs on complete medium the isolate may represent an auxotrophic mutant. Less than 1 per cent of the colonies tested in this random isolation procedure is likely to be auxotrophic.

A more efficient method for the isolation of auxotrophic mutants

[11] Lederberg, J., *Methods in Medical Research, 3*:5, 1950.

is the delayed enrichment or layer plating technique. In contrast to the random isolation method in which one colony is tested at a time, the delayed enrichment method permits the simultaneous screening of several hundred colonies. A thin layer of unseeded minimal agar is poured into a Petri dish and, after it has solidified, a second layer of minimal agar containing 200 to 300 cells from a primarily prototrophic population is added. A third layer of minimal agar is then poured over the solidified second layer. Following incubation, prototrophic cells will give rise to visible colonies which will not spread because of the unseeded bottom and top layer. Mutant cells (auxotrophs) will fail to develop into visible colonies because their growth-requirements are not met by the minimal medium. However, if a fourth layer consisting of complete medium is now poured on top of the plates the required growth factors will diffuse through the other agar layers and stimulate growth of the auxotrophic cells into visible colonies. Several hours later the auxotrophs can be identified by the smaller size of their colonies. Identification of these mutant colonies can be simplified by a trick developed by Horowitz.[12] Following the initial period of incubation in minimal medium a contact print is made by placing the plate on a sheet of photo-paper and exposing it to light. After development, it will show all colonies which grew during the primary incubation period as white spots on a black background. Following the pouring of the complete agar and a second incubation period the plate is placed on the developed print in exactly the same position as during the preceding exposure. All colonies that developed during the primary incubation now lie on a white spot and a blue light placed underneath the plate will permit the light to penetrate giving "primary" colonies a blue appearance; "secondary" colonies (probable biochemical mutants) will not show up blue because they are lying on a dark background but will appear yellow by reflection if a yellow light is placed above the plate.

As illustrated in Figure 23, replica plating provides another simple method for the isolation of auxotrophic mutants.

The most efficient method so far developed for the isolation of biochemical mutants is the "penicillin method" discovered simultaneously by Lederberg[13] and Davis[14] (Fig. 34). This method is applicable to all bacterial species sensitive to penicillin and depends

[12] Personal communication.

[13] Lederberg, J. and Zinder, N., J. Am. Chem. Soc., *70*:4267, 1948.

[14] Davis, B. D., J. Am. Chem. Soc., *70*:4267, 1948.

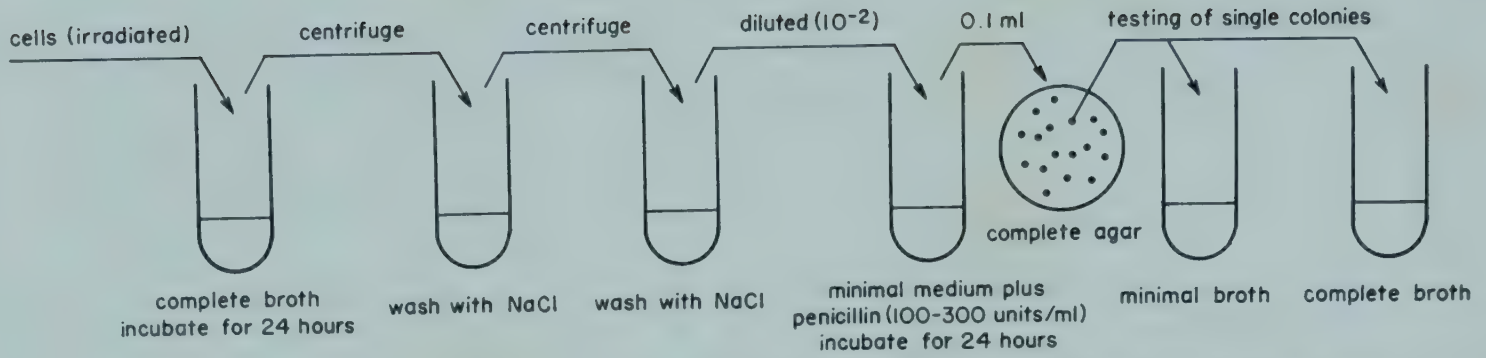


Fig. 34. Isolation of auxotrophic mutants by the penicillin screening method. The length of incubation periods indicated are for *E. coli* and may require modification for work with other species.

upon the fact that penicillin is bactericidal only for dividing cells. Hence when a population of prototrophs is suspended for 12 to 24 hours in minimal medium containing bactericidal concentrations of penicillin most of the prototrophs will be killed because they divide in this medium. At the same time any auxotrophic mutants present will fail to grow in this deficient medium and, therefore, will survive despite the presence of penicillin. Subsequent plating of the penicillin-treated population onto complete medium will yield a high proportion of mutant, auxotrophic colonies as determined by their inability to produce growth when subcultured on minimal media. However, for some as yet unknown reason, as many as one third of the apparent mutants may turn out to be prototrophs after renewed subculturing. Despite this erratic behavior of some isolates, the "penicillin method" represents a most useful tool for the isolation of auxotrophs and certain modifications that increase its efficiency even further have been developed recently.[11]

The most important modification has been developed by Adelberg and Myers.[15] Instead of use of liquid media, the whole procedure is carried out in a solid medium. First, a layer of minimal agar containing irradiated cells is poured into plates. After a few hours of incubation a layer of minimal agar containing penicillin is added. The penicillin is inactivated after 24 hours of incubation by the pouring of an additional layer of minimal agar containing penicillinase. The plates are reincubated and finally a layer of complete agar is poured on top of the previous layers. Renewed incubation will now promote the growth of auxotrophic mutants into colonies. The yield of auxotrophic mutants is very high in this procedure, and whereas in the liquid cultivation method several of the phenotypically identical auxotrophs may have originated from one original mutant cell, in Adelberg and Myer's procedure each auxotrophic colony corresponds to one mutational event.

The penicillin method also lends itself to the isolation of specific classes of mutants; this is accomplished by adding to the minimal agar layer, or to the penicillin-containing minimal liquid medium, specific growth factors which will support the growth of undesired mutants, thereby eliminating them along with the prototrophs.

Assaying growth requirements

Once mutants with increased growth requirements have been isolated, the specific nature of the requirement can be ascertained

[15] Adelberg, E. A. and Myers, J. W., Microb. Genetics Bull. #6, 1952.

by several methods. One method consists of inoculating the mutant into minimal medium to which one of the following complex supplements has been added: (1) vitamin-free hydrolyzed casein which will support growth if the mutant requires a specific amino acid; (2) a mixture of water soluble vitamins which will support growth if one of these vitamins is required; (3) hydrolyzed yeast nucleic acid which will satisfy growth requirements for purines, pyrimidines, nucleosides and nucleotides; and (4) yeast extract which will stimulate the growth of certain vitamin-requiring mutants. Once the growth-response of a mutant to any of these general groups has been ascertained, the specific requirements can be determined by testing for growth in minimal media supplemented with either specific amino acids or specific vitamins, etc. Detailed suggestions for such tests have been compiled by Lederberg.[11]

Beijerinck's *auxanographic method* is another helpful technique for the characterization of biochemical mutants. The cells to be tested are incorporated into a layer of minimal agar and individual supplements, either in the form of crystals or as drops of a solution, are placed on the surface of the agar. The response to a specific supplement can then be recognized by growth in the area where the needed growth factor diffused into the agar. This method also permits the identification of complex requirements yielding growth in areas where the boundaries of two or more supplements overlap.

The replica plating technique (p. 55) also can be used for assaying the nutritional requirement of auxotrophic mutants.

Polyauxotrophic strains

From auxotrophic mutants displaying one added growth requirement, e.g., leucine, additional mutants may be isolated showing still other growth-requirements, e.g., from a leucine-requiring mutant one might isolate a mutant requiring leucine plus arginine. In this manner polyauxotrophic strains can be "built up" and such strains have proven to be of considerable experimental value in certain studies (see p. 187).

Reverse mutations

Auxotrophic mutants are apt to revert to nutritional competence, i.e., the prototrophic characteristics may be restored by mutational steps. Thus prototrophic mutants, which no longer require the

formerly needed growth substance, may occur in a population of auxotrophs and may selectively replace the auxotrophic cells. Such changes from populations requiring specific growth-factors to populations capable of growing in minimal media have all the aspects of adaptation in the sense of "training" bacteria to dispense with formerly needed growth factors. However, experimental studies of the type discussed in Chapter 4 indicate that this is a case of mutation and selection. The actual nature of these reverted mutants may differ. Some have been claimed to represent true reverse mutants in which the original prototrophic condition is restored not only physiologically but also genetically. Others represent only apparent reversions, that is, they involve mutants which phenotypically resemble the prototrophic parent type but in which the activity of the mutated gene has been altered by an additional *suppressor mutation*. Suppressor mutations are well known from genetic studies with higher organisms and fungi but limited evidence indicates that they occur in bacteria as well.[16] A suppressor mutation involves a change of a gene that is different from the one controlling the nutritional deficiency, the mutated suppressor gene, in turn, can alter the biosynthetic processes that are affected by the unrelated gene. To choose an arbitrary model for such a phenomenon let us assume that a certain gene mutated, thereby causing a requirement for leucine. If this reverted to its original state, the requirement for leucine would disappear due to a reverse genetic change. However, in the case of a suppressor mutation the gene controlling the leucine requirement, let us call it gene #1, remains unaltered but an entirely different gene, gene #2, changes or mutates and thereby becomes capable of suppressing the action of gene #1. It is also possible that the mutated gene #2 causes an alteration of metabolic pathways in such a manner that leucine is no longer required for growth, thereby masking the continued presence of the unaltered gene #1. These apparent reversions, leading to mutants that merely mimic the original parent type but are not genetically identical with it are of some importance for considerations which we shall discuss later under the subject of changes in antigenic characteristics. In any event, prototrophs may occur in auxotrophic populations and it is, therefore, important to maintain stocks of biochemical mutants under conditions which

[16] Witkin, E. M. and Kennedy, F. L., Amer. Nat., *85*:141, 1951.

would give such reverse mutants the least chance of establishing themselves once they arise. A complete medium which adequately supplies the growth requirements of the auxotrophic mutant should be chosen for maintaining such cultures. If prototrophs are found in auxotrophic stock-cultures, they can be eliminated fairly rapidly with the help of the penicillin-technique described above.

Blocking of biosynthetic steps and syntrophism

Auxotrophic mutants displaying a similar requirement are not necessarily identical because mutations may affect different steps in one chain of biosynthetic processes. These problems have been extensively investigated in studies with *Neurospora* mutants but they have been shown to apply as well to biochemical mutants of bacteria.[17] For example, it is known that part of the synthesis of tryptophan proceeds through the following steps, all of which can be carried out by prototrophic cells: $X \xrightarrow{1}$ anthranilic acid $\xrightarrow{2}$ indole + serine $\xrightarrow{3}$ tryptophan.

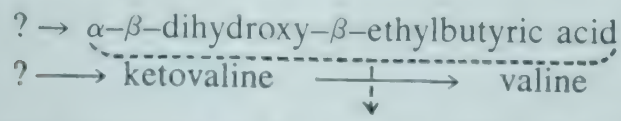
A mutation blocking step 3, presumably by affecting the activity of the enzyme catalyzing the condensation of indole and serine, will lead to a requirement for tryptophan which can be repaired only by the addition of tryptophan itself. However, another mutation which blocks step 2 will produce auxotrophs whose requirement for tryptophan can be compensated for by the addition of indole or tryptophan. Similarly, a mutation that interferes with step 1 may cause a tryptophan requirement which can be remedied by the addition to the minimal medium of either anthranilic acid, indole, or tryptophan. Blocking at different steps is usually due to the action of different genes. When such a mutational block occurs, the precursor tends to accumulate, e.g., anthranilic acid will accumulate if step 2 is blocked in the above scheme. The accumulated substance may be excreted into the medium, frequently in sufficient quantities to supply the needs of another mutant blocked at an earlier step. This second mutant can then grow and supply the final product to the first. This mutual feeding is known as *syntrophism*. Thus two mutants with similar requirements, each incapable of growing by itself in a minimal medium, may grow when placed together in minimal media because the accumulating intermediate

[17] Davis, B. D., Experientia, *6*:41, 1950.

of one mutant may be able to stimulate growth of the other which in turn supplies the end product to the first. For example, two tryptophan-requiring mutants, one blocked at step 3 of the above scheme, the other at step 2, may both grow when placed on a minimal medium. Syntrophic growth also can be employed for testing whether two mutants with similar nutritional requirements are blocked at different steps in the synthesis of the growth factors. However, Davis[17] has pointed out that absence of syntrophism between two mutants with a common requirement does not always prove that they are blocked at the same enzymic site, "since precursors may fail to accumulate because of instability, diversion along an alternative path, or the inability of the cell to build up a concentration adequate for excretion."

Interactions between different biosynthetic pathways

Studies with auxotrophs have uncovered a particularly interesting case of internal inhibition between two separate pathways, illustrating the complexity that may exist in the interrelations among various biosynthetic paths. Bonner[18] isolated a mutant of *Neurospora*, and Davis[17] later isolated a similar mutant of *E. coli*, which showed a double requirement for both isoleucine and valine. Further analysis of these mutants showed that a single block in isoleucine synthesis between the dihydroxy acid[19] and isoleucine was responsible for the double requirement. The accumulated isoleucine precursor appears to act as an inhibitor for the conversion of ketovaline to valine which normally takes place in the separate pathway of valine synthesis:



In this manner a single mutation initially affecting only one enzyme system may lead to an effect on two separate pathways. Similar cases, some of them indicative of even more complex physiological interactions among separate biosynthetic pathways or within a single biosynthetic sequence, have been found in studies with other auxotrophic mutants.[17]

[18] Bonner, D., J. Biol. Chem., *166*:545, 1946.

[17] Adelberg, E. A., Bonner, D. and Tatum, E. L., J. Biol. Chem., *190*:837, 1951.

Some applications of auxotrophic mutants

The ability of auxotrophic mutations to block biosynthetic reaction chains at different steps has made them extremely useful for studies on the biosynthesis of growth factors. Although a great deal of this work has been carried out with the help of *Neurospora* mutants, bacterial mutants have also been used widely.[17, 20] The principal method in such studies is the identification of intermediates which are capable of substituting for the requirement of an end product; e.g., the ability of anthranilic acid and indole to satisfy the growth requirements of certain tryptophan-requiring mutants indicated that these compounds must play a role in the biosynthesis of tryptophan. The sequence of the biosynthetic steps is reflected by the requirement of different mutants. For example, if two mutants requiring tryptophan are found and both can grow on indole but only one can grow on anthranilic acid, it follows that anthranilic acid is the precursor of indole in the biosynthesis of tryptophan.[20] Similarly, auxotrophic mutants have been used in the analysis of biosynthetic steps involved in the light production by luminous bacteria[21] and in nitrogen fixation by *Azotobacter*.[22]

So far, only limited use has been made of auxotrophic mutants in another field where they might contribute important information, namely, in the analysis of factors and biosynthetic steps involved in the development of antigenic and virulence characteristics of bacterial pathogens. Some work along these lines, to be described in Chapter 7, has been done with *Salmonella* species.

Experimentally obtained auxotrophic mutants can be used for biologic assays of various amino acids, vitamins and nucleic acid derivatives, just as naturally occurring exacting mutants of Lactobacilli have been used for such purposes. The increasing list of auxotrophs of *E. coli* (see Microbial Genetics Bulletin issued by the Carnegie Institution, Cold Spring Harbor, Long Island, New York) furnishes an impressive assortment of material for biologic assays, including the analysis of quantitative and qualitative production of specific metabolites by various bacterial species and antigenic variants.

Nutritional requirements can serve as excellent "markers" in

[20] See chapter by Gale, E. F. in *Bacterial Physiology*, edited by Werkman and Wilson, Academic Press, Inc., New York, 1951.

[21] McElroy, W. D. and Farghaly, A. H., Arch. Biochem., *17*:379, 1948.

[22] Wyss, O. and Wyss, M. B., J. Bact., *59*:287, 1950.

studies on population dynamics[23] and in the analysis of recombination and transformation problems. We shall discuss their use in such studies in more detail in later chapters.

FERMENTATION MUTANTS

Variability in fermentative characteristics represents one aspect of bacterial variation that has been well recognized by bacteriologists since the days of Pasteur. In fact, one of the earliest contributions to bacterial genetics, dating back to 1907, had as its subject changes in fermentative characteristics of *E. coli* strains.[24] Massini showed in these early investigations that a non–lactose-fermenting

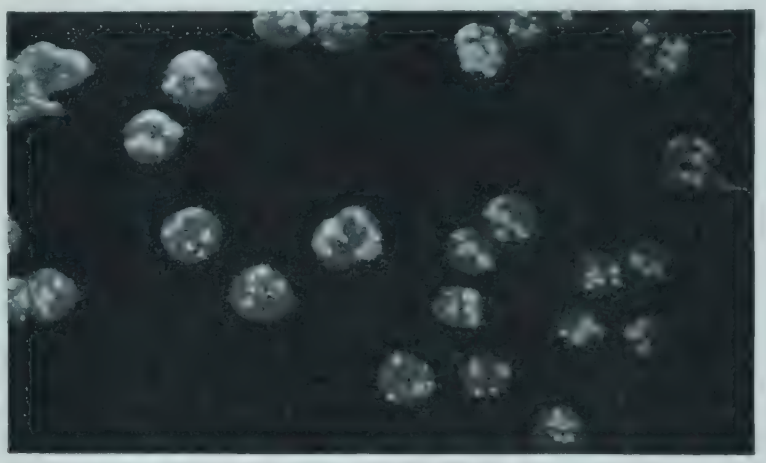


Fig. 35. Lac^+ papillae on lac^- colonies of *E. coli*. (Parr.)

strain (nowadays labelled lac^-) gave rise to lactose-fermenting (lac^+) variants which could be recognized by cultivating the cells on lactose agar containing an indicator that turned red when acid was formed. Thus lac^- cells appeared white, whereas lac^+ cells, which fermented the sugar to acids, appeared red. When lac^- cells were plated on an indicator medium, white colonies first developed but upon continued incubation red (lac^+) papillae appeared (Fig. 35). Upon subculturing from such papillae stable lac^+ strains could be isolated. Massini recognized such variants as mutants and later work by Lewis[25] revealed that lac^+ mutants occurred at a constant frequency of approximately one lac^+ cell per 100,000 lac^- cells. Various other fermentation mutants have since been studied, espe-

[23] Ryan, F. J. and Schneider, L. K., J. Bact., *56*:699, 1948; *58*:181, 1949; *58*:191, 1949; *58*:201, 1949.

[24] Massini, R., Arch. Hyg., *61*:250, 1907.

[25] Lewis, J. M., J. Bact., *28*:619, 1934.

cially with members of the colon-typhoid-dysentery group, e.g., mutants differing in the utilization of citrate[26] and maltose.[27]

In comparison with mutations to auxotrophy or resistance to antibacterial agents, the mutation rate involving changes in fermentative abilities is usually high, about 1×10^{-7} to 1×10^{-5} per generation. Why the stability of fermentative characteristics should be so much less than that of many other characteristics is entirely unknown. We are faced with a similar lack of stability in the case of pigmentation and many antigenic mutants. It is possible that in these cases of relative instability control by cytoplasmic factors rather than *sole* control by nuclear factors may be involved (see p. 126 for further discussion of cytoplasmic inheritance). On the other hand, recombination studies with *E. coli* (p. 190) have clearly demonstrated that many fermentative characteristics are controlled by nuclear genes. The real cause of relative instability in the characteristics cited above, therefore, must remain unresolved for the time being.

Although changes in fermentative abilities are mutational in nature, in many cases the ability of bacteria to produce the specific enzyme requires the presence of a specific substrate. This phenomenon is known as *enzymatic adaptation* (see Chapter 10). It may be distinguished from mutational changes by the fact that it can occur without multiplication of cells and involves changes in all cells of a competent bacterial population. In these cases the phenotype cannot be expressed unless specific environmental conditions prevail; however, the potential capacity for this phenotypic change is under genic control. Some mutants manifest maximum fermentative abilities immediately upon contact with the substrate that they can utilize, without displaying the time lag apparent in the case of enzymatic adaption. Such observations have led to a distinction between adaptive and constitutive enzymes but it is doubtful whether such a distinction has more than descriptive value.[28] Many investigators now believe that the differences between such mutants, or between the enzyme systems involved, may be quantitative rather than qualitative. In other words, the presence of the substrate may

[26] Parr, L. W. and Simpson, W. P., J. Bact., *40*:467, 1940.

[27] Doudoroff, M., Hassid, W. Z., Putnam, E. W., Potter, A. L. and Lederberg, J., J. Biol. Chem., *179*:921, 1949.

[28] Spiegelman, S., in *The Enzymes*, edited by Sumner and Myrbäck, Academic Press, Inc., New York, *1*:267, 1950.

always be required to activate a particular enzyme, but the rate at which such activation occurs may be sufficiently fast to escape detection, or the level of the specific enzyme in non-adapted cells may be high because the cells can manufacture the required substrate from related carbohydrate sources present in the medium. To illustrate the last point, let us refer to the enzymes responsible for the utilization of galactose and glucose. The first, galactozymase, is not found in the cell unless galactose is present in the medium, whereas the second, glucozymase, may be formed in the absence of glucose in the medium. However, polysaccharides of glucose and intermediates of glucose metabolism have been found in cells which have been metabolizing galactose and can account for the presence of glucozymase even though the specific substrate, glucose, is lacking in the medium.[28]

In view of these facts it is possible to apply a general definition to all fermentation mutants: They represent mutants which arise spontaneously in small numbers in a population consisting primarily of cells with different fermentative capacities; they can maintain their genetic competence for fermentative characteristics in the absence of the specific substrate, but may require appropriate conditions to express their fermentative ability.

A mutation endowing a cell with the capacity to ferment a new carbohydrate source may not always affect the specific enzyme system directly. For example, it has been suggested that the difference between lac$^-$ and lac$^+$ cells consists in different permeability to lactose,[29] and Monod[30] has described a strain of *E. coli* which grows slowly in galactose because its galactozymase activity is inhibited by some product of galactose utilization. A galactose-positive mutant isolated from this strain produces less of this enzyme inhibitor and, therefore, grows very fast in galactose.

Fermentation mutants are useful tools for genetic studies with bacteria. They can be readily recognized by plating on indicator media such as eosin-methylene blue (EMB) agar or triphenyltetrazolium agar[11] containing the appropriate carbohydrate. On EMB agar colonies that ferment the carbohydrate produce a dark purplish color; non-fermenters fail to do so. On tetrazolium-agar non-fermenters appear bright red and fermenters remain uncolored. The reaction is sufficiently sensitive to permit the easy identification of

[29] Deere, C. J., Dulaney, A. D. and Michelson, I. D., J. Bact., *37*:355, 1939

[30] See Lwoff, A., Cold Spring Harbor Symp. Quant. Biol., *11*:139, 1946

non-fermenter sectors in fermenter colonies, or vice versa. This simplicity of identification makes fermentation mutants extremely useful for studies on recombination (p. 190) and for population studies where the fate of two easily distinguished types is determined. A specific example of the application of this "marker" technique has already been cited in connection with studies on the delayed appearance of mutants (p. 76).

PIGMENTATION MUTANTS

Another group of biochemical mutants that has received some attention due to the ease of identification is the one involving changes in pigmentation. For example, both Bunting[31] and Lincoln[32] have made extensive use of differences in pigment production displayed by various *Serratia marcescens* and *Phytomonas stewartii* mutants, respectively, to analyze mutation rates and phenomena of selection. Their investigations have supplied substantial evidence indicating that changes in pigmentation can be attributed to mutations. Pigmentation, however, is also a characteristic that is subject to temporary modifications due to environmental changes. For example, changes in the medium such as a shift in pH can cause changes in pigmentation. In contrast to mutations, such temporary modifications affect all members of a homogeneous population and will last only as long as the changed environment persists. Pigmentation mutants, on the other hand, arise as individual variants among a large number of genetically different parent cells. Thus, it is important to distinguish between pigmentation variants due to mutation and those caused by temporary modification. The use of constant environmental conditions is one of the necessary requisites in studies on pigmentation.

Mutation rates of most pigmentation mutants thus far studied differ widely and can be very high; rates varying from 1×10^{-3} to 1×10^{-7} have been observed. The cause of the relative instability of some of these mutants is still unknown. The possible involvement of cytoplasmic factors has been suggested in one case[33] but has not been proven. In general, the considerations expressed in the discussion on the stability of fermentation mutants also apply here.

[31] Bunting, M. I., Cold Spring Harbor Symp. Quant. Biol., *11*:25, 1946.
[32] Lincoln, R. E., J. Bact., *54*:745, 1947.
[33] Bunting, M. I., Labrum, E. L. and Hemmerly, J., Amer. Natural., *85*:331, 1951.

CHAPTER 7

Representative Types of Mutants: II

In the analysis of variable properties that are primarily associated with pathogenic bacteria, most investigators have paid special attention to changes in colonial morphology, antigenic characteristics, and virulence. There is good evidence that changes in these properties are usually caused by spontaneous mutations and that most mutations affecting one of these characteristics also affect the other two characteristics. For example, a change in virulence is usually, though not necessarily, associated with changes in colonial morphology and antigenicity. Similarly, environmental conditions capable of modifying, for example, antigenic properties are also likely to cause temporary changes in colonial morphology and virulence. Because of the high degree of correlation between changes in these three properties, much of the information obtained in studies with one tends to overlap with information directly applicable to the variation of the two other properties. For this reason, it will be convenient to consider first the more specific aspects of each class of mutants and to refer subsequently to certain general problems, including mutation rates and the possible involvement of cytoplasmic factors.

MUTATIONS AFFECTING COLONIAL MORPHOLOGY

Detection

Cells placed singly or in groups on solid media will develop into visible colonies that may differ in appearance not only between species but within species. Many factors may contribute to such differences in colonial morphology. Among them are the morphology and the surface structure of the individual cells and their arrangement within the colony. The latter is partly determined by the manner in which the cells move following division on solid media. For example, Bisset[1] has shown that when cells grow under

[1] Bisset, K. A., *The Cytology and Life-History of Bacteria*, Livingstone, Ltd., Edinburgh, 1950.

the severe mechanical restraint imposed by a solid medium "smooth bacteria grow and elongate against the pressure of the surrounding medium. When separation is complete, the daughter cells," occurring singly (see p. 48), "may be forced back again, side by side, by the elasticity of the agar." In contrast, "rough bacilli remain attached at the point of division (see p. 48) and the filament,

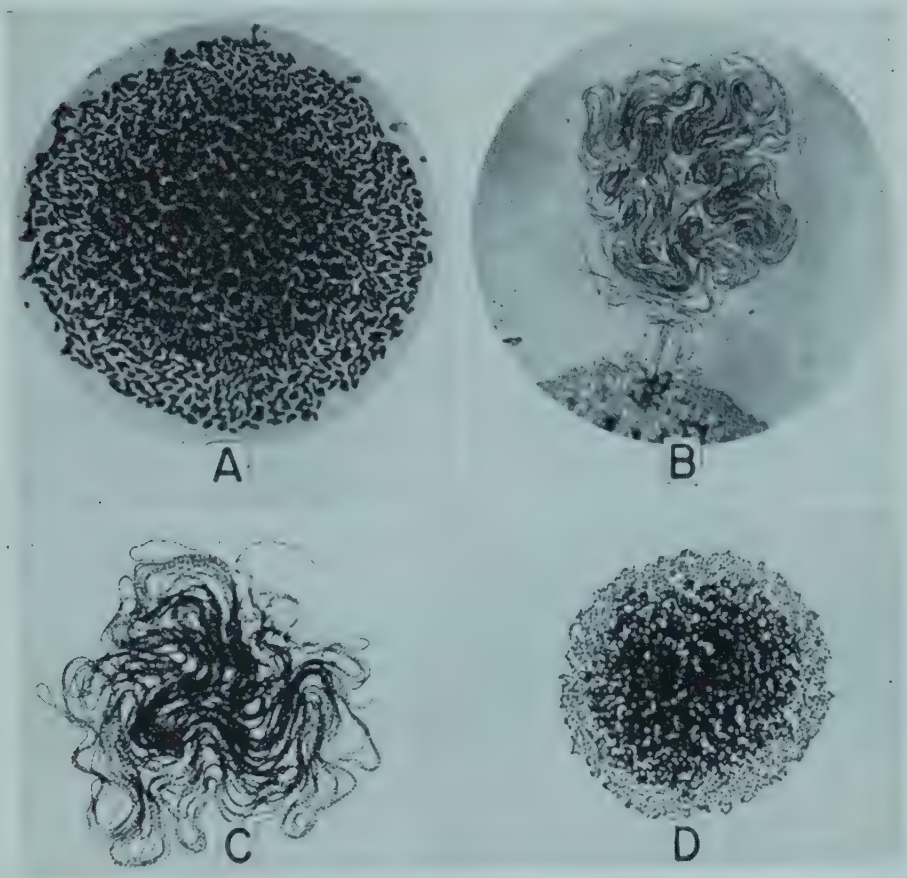


Fig. 36. Smooth and rough colonies. *A*, Smooth colony of *E. coli*, impression preparation, × 500; *B*, rough colony arising from the perimeter of a smooth colony of *Shigella dysenteriae*, × 300; *C*, long-chained rough colony of streptococcus, × 300; *D*, short-chained smooth colony of streptococcus, × 300. (Photos by Bisset, reproduced by courtesy of the Journal of Pathology and Bacteriology and the Journal of General Microbiology.)

constrained in the same manner, is compressed concertina-fashion, bending at the points of division" (Fig. 36).

Many differences in colonial morphology can be detected with the unaided eye; additional differences become apparent if a dissecting microscope is used for inspecting colonies. However, the most sensitive system for the detection of otherwise inapparent

differences in colonial morphology consists of microscopic observations with the help of obliquely transmitted light[2] (Fig. 37). In addition to this critical illumination an appropriate transparent medium is required, because many differences may be inapparent on one medium but show up clearly on other media. To illustrate what may be accomplished with such techniques, Figure 38 shows some of the colonial types of *Bacterium tularense* which have been detected on glucose-cysteine-peptone agar with the help of the

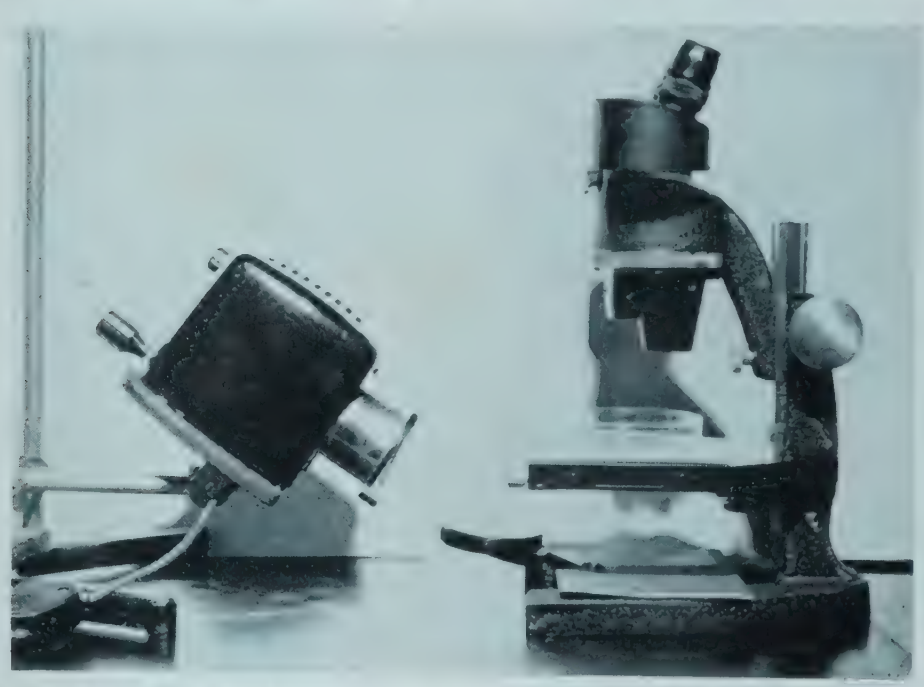


Fig. 37. Arrangement of mirror and lighting source for inspection of colonial morphology by obliquely transmitted light.

oblique lighting technique.[3] Such differences as those visible in Figure 38 are of considerable value for recognizing differences in antigenicity and virulence (see p. 123), but remained undetected until the proper lighting and medium were employed. In studies with various enteric organisms and with *Brucella* species a nutrient medium containing 1 per cent dextrose and 2 per cent glycerol, 2–1 agar,[2] has been found particularly suitable for the differentiation of colonial types. In some other studies the use of oblique lighting and MacConkey's agar has proved helpful (see p. 111). Unfortunately, most workers have not employed such improved techniques, but, nevertheless, the literature on differences in colonial

[2] Henry, B. S., J. Inf. Dis., *52*:374, 1933.

[3] Eigelsbach, H. T., Braun, W. and Herring, R. D., J. Bact., *61*:557, 1951.

morphology between species and within species is very voluminous.[4, 5] The major reason for this intense study of colonial characteristics is the frequent association between changes in colonial

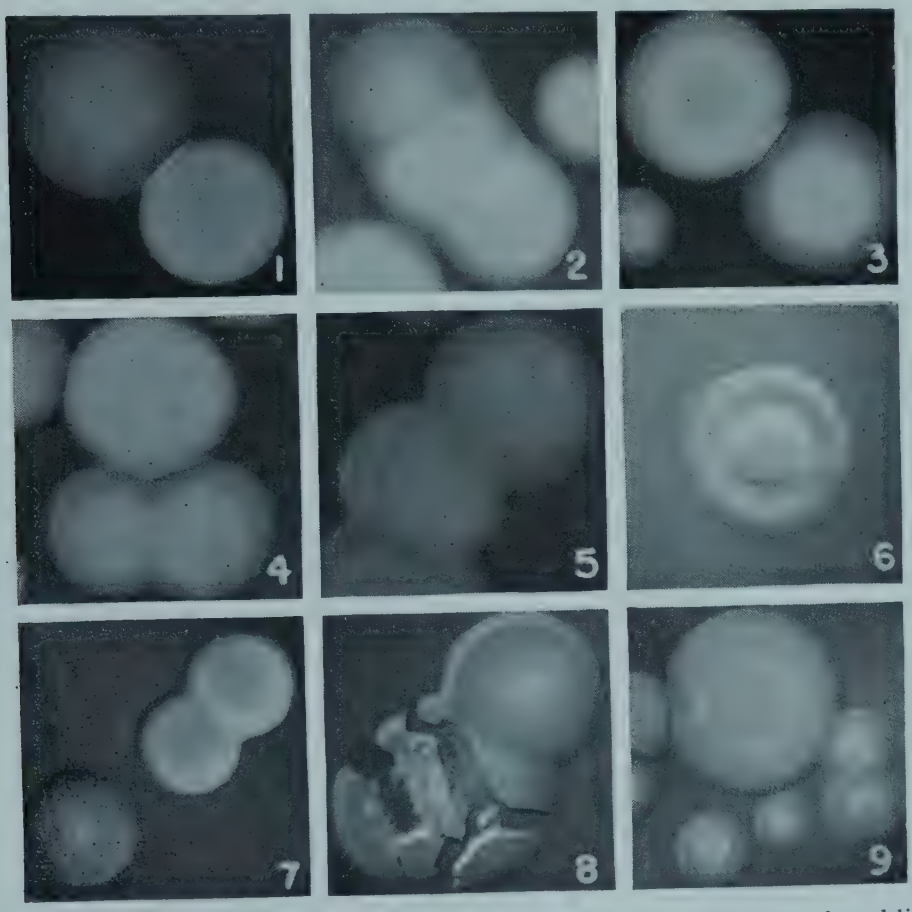


Fig. 38. Various colonial types of *B. tularense* as revealed by the oblique lighting technique. The majority of colonies shown give smooth acriflavine reactions. Non-smooth acriflavine reactions are produced by the two lower colonies in *4*, the upper one in *7*, the smaller colonies in *9*, and the colonies shown in *8*. The latter are extremely brittle and, when touched with a wire, break into several pieces as illustrated in the lower portion of the photograph. (From Eigelsbach, Braun, and Herring.[3])

characteristics and changes in other less readily determined characteristics such as virulence, antigenicity and immunogenic potency.

Colony types

Several characteristic groups of colony variants have been noted within most bacterial species and they have been referred to as

[4] Hadley, P., J. Inf. Dis., *40:*1, 1927; J. Inf. Dis., *60:*129, 1937.
[5] Braun, W., Bact. Rev., *11:*75, 1947.

smooth (S), rough (R), mucoid (M), and intermediate (I). Smooth colonies are usually round and glistening; rough colonies have a granular or wrinkled appearance with a tendency towards irregular margins; mucoid colonies are commonly round, transparent, and because of the mucopolysaccharide capsule of their constituent cells, have a slimy consistency; intermediate colonies, which are usually round, opaque or slightly granular, have an appearance intermediate between smooth and rough colonies. Careful inspection, especially with oblique lighting, has revealed the occurrence of an enormous number of different colonial types within each of these major groups. For example, various types of intermediate, such as I_1, I_2, I_3, or of smooth, such as S′, S″, etc., have been described in the literature. In addition, other groups of colony types frequently described are the so-called G type consisting of minute colonies frequently containing filterable elements, a dwarf (D) type characterized by small colonies usually containing diphtheroid forms, and the L-type, containing pleuropneumonia-like organisms (see p. 47). In a few species still other colonial types have been described; for example, virulent hemolytic streptococci occur in a form designated "matt." In many species mutants with unchanged cellular morphology have been described which give rise to small colonies. Such "small colony variants" appear to have a lowered metabolism[6] or a partial requirement for certain growth factors.[7] Accurate classification into the above major groups of colonies by means of visual inspection cannot always be accomplished, since no objective standard can be applied and all visual observations are subjective in nature. However, as long as it is recognized that changes in colonial morphology do not represent definite steps in a life-cycle as envisaged by some investigators,[4] but are merely indicators of undirected, spontaneous changes in other associated characteristics,[5] it becomes relatively unimportant to identify each colonial form with one specific type.

The significance of changes in colonial morphology

Despite the absence of objective criteria for the identification of specific colony types, the approximate classification of colonial types of pathogens into general groups, represented by S, R, or M, has proved very useful in relation to the natural occurrence of

[6] Colwell, C. A., J. Bact., *52*:417, 1946.
[7] Gillespie, W. A., J. Path. Bact., *64*:551, 1952.

certain colony types. Thus the pathogenic forms found most frequently in nature are of one colonial type only, usually the S type (for example: the Salmonella species) or the M type (for example: the pneumococcus species as classified according to Dawson's terminology[8]); a few species, such as anthrax, have usually been found as R types in primary isolations. When such isolates are grown under laboratory conditions, population changes involving the establishment of mutants with altered colonial morphology are usually observed. This phenomenon also has been referred to as *bacterial dissociation*. The observation that such *in vitro* population changes usually proceed from S → R → M or from M → S → R has led to the claim that this represents a definite life-cycle of the culture.[4] It is now known that such trends are due merely to the progressive replacement of the initial type by mutants possessing certain properties which endow them with successively higher selective value and with which changes in colonial morphology may be associated (see Chapter 8). Similarly, the "normal" type found in natural habitats merely represents a particular mutant in which the properties causing a specific colony type are associated with other properties that favor the cells' survival in nature. In the case of pathogenic bacteria some of these properties can be identified with the presence of certain surface components of the cells which render the bacteria more resistant to the defense mechanism of the host and thereby contribute to greater virulence. The same surface components may also determine antigenic properties of the cell in addition to the morphology of the colony of which they are a part. Changes in colonial morphology, therefore, are primarily useful as indicators of changes in associated characteristics.

Colonial morphology as indicator of changes in resistance and growth requirements

Undoubtedly most of the smooth, rough, mucoid, etc., changes are in the nature of a biochemical mutation. Even though we know very little about the steps involved in the biosynthesis of surface antigens or factors associated with virulence, any change in these characteristics must involve some change in a biosynthetic process. Changes of this nature, as we have just seen, may be reflected by changes in colonial morphology and, therefore, it is not surprising to learn that such biosynthetic changes as those occurring in

[8] Dawson, M. H., J. Path. Bact., *39*:323, 1934.

auxotrophic mutants and resistant mutants of *E. coli* can also cause minor but significant changes in colonial morphology.[9] To detect such changes it is necessary to employ the oblique lighting system and, preferably, a medium containing a dye. Thus, inspection of the colonial morphology of more than 20 different *E. coli* auxotrophs cultured on MacConkey's agar revealed distinctly different colonial

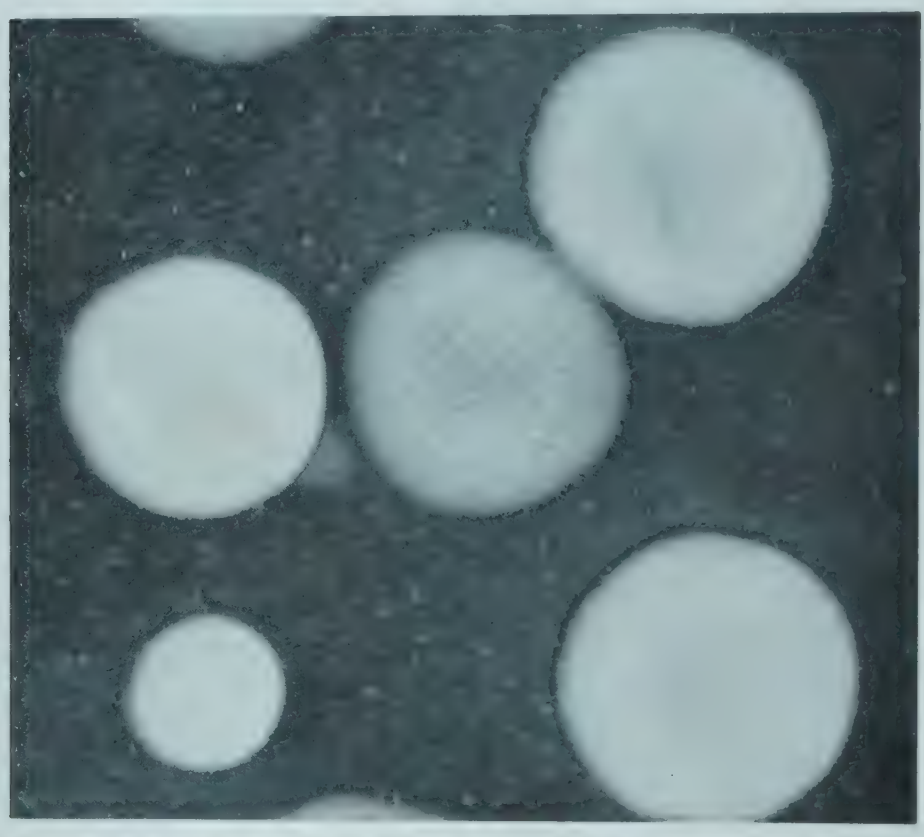


Fig. 39. Four S^s (threonine-susceptible) colonies of *Salmonella typhimurium* surrounding an S^t (threonine-resistant) colony. (From Page, Goodlow, and Braun.[29])

types for almost every one of the mutants tested. Differences between radiation-resistant and radiation-sensitive mutants as well as between streptomycin-sensitive, -resistant, and -dependent mutants were also observed. Such easily detected differences are useful in at least three ways: (1) they permit the simple and relatively rapid recognition of characteristics such as radiation-resistance, which would otherwise be more difficult to detect; (2) they allow the rapid determination of the fate of two mutants with different growth requirements or with absolute differences in resistance during growth

[9] Braun, W. and Lewis, K. H., Genetics, *35*:97, 1950.

in a non-selective medium; for example, competition between streptomycin-resistant and streptomycin-sensitive mutants can be tested without the use of streptomycin as a screening agent which would eliminate one of the mutant classes; (3) they make possible a rapid determination of the heterogeneity or homogeneity of a culture. Although, as stated above, characteristic colonial differences can be found among most mutants so far tested, it has also been recognized that different colonial types may occur in a mutant

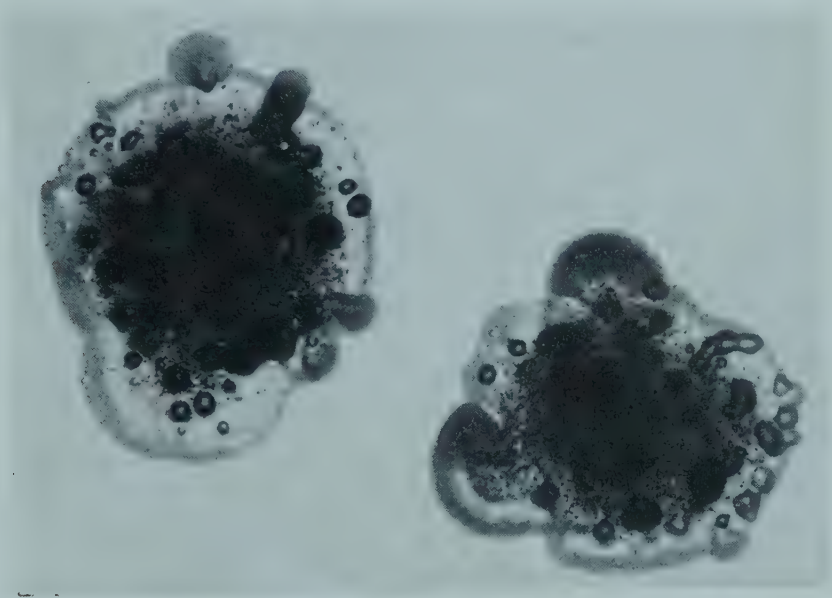


Fig. 40. Papillae (daughter colonies) on colonies that developed from B/r cells of *E. coli* on MacConkey's agar. Incubation time—3 days at 37° C. plus 3 days at room temperature. Magnified 8 times. (V. Bryson.)

culture apparently homogeneous for a given characteristic, e.g., histidine requirement. In such cases it must be assumed that histidine-requiring mutants differing in some other characteristic, such as growth rate or phage-resistance, are coexisting in the culture. The use of one stable colonial type isolated from such a culture is preferable for experimental work.

The aforementioned use of MacConkey's medium has also revealed significant, previously unrecognized, colonial differences in species other than *E. coli*. For example, it was found in studies with *Salmonella typhimurium* that antigenically identical threonine-resistant and threonine-sensitive mutants, differing in virulence, can be detected with ease following growth on MacConkeys agar (see Fig. 39 and p. 169). Other observations have shown that similar

significant differences between antigenically similar but immunogenically different types of various enteric organisms can be observed on MacConkey's agar. One note of caution in regard to the use of MacConkey's agar is in order. It has been noted that on this medium certain mutants of *E. coli* will develop into colonies showing a multitude of papillae (secondary colonies) after 48 hours of incubation (Fig. 40). Similar changes do not occur on nutrient agar. The actual cause for this instability, involving either selective growth of new mutants or their increased occurrence, is still unknown. Naturally, subculturing from such heterogeneous colonies would result in heterogeneous cultures. Therefore, in cases like this it may be advisable to use the appearance on MacConkey's agar as a useful indicator but to choose for subculturing corresponding colonies from parallel plates with nutrient agar, the latter preferably produced with the help of the replica plating technique.

Environmental effects upon colonial morphology

The available evidence indicates that changes in colonial morphology may be produced by environmental influences as well as by mutations. Several criteria can help in determining whether an alteration in colonial morphology is due to an environmentally induced temporary modification or a mutational change. (1) If an initially homogeneous population consisting of one colonial type is subcultured under altered environmental conditions and all developing colonies show a different appearance, a temporary modification must be suspected. The return of such a population to the original environmental conditions should restore the original phenotype. In contrast, mutational changes are likely to be involved if subcultures from a population of uniform colonial appearance yield several different appearing colonies in addition to colonies predominantly of the original type. (2) The occurrence of two or more different colonial types in a population kept under identical conditions, e.g., on the same plate, is indicative of genetic differences between the colonies under observation, provided that the colonies are of equal age and evenly distributed over the plate; crowded growth or differences in age of colonies can lead to differences in appearance which are not genetically controlled. Those differences which are not due to age or crowding may become inapparent when the same population is grown under different environmental conditions. Of course, this does not indicate a dis-

appearance of the genetic differences between the cells which previously led to dissimilar colonial types; it means merely that such differences cannot be detected phenotypically. Return to the original conditions should restore the differential appearance. For example, on minimal synthetic agar phage-resistant mutants of *E. coli* produce a colony type different from that produced by phage-sensitive cells. These differences become inapparent when the two types of cells are permitted to grow on a complex agar medium. It also happens frequently that specific environmental conditions cause changes in colony morphology which copy in all details the appearance of certain colonial types which can arise by mutation. Such modifications can be classified as phenocopies (see p. 3). For example, in studies with *Lactobacillus spp.* it was observed that cells which normally develop into rough colonies on an adequate tomato juice agar will produce smooth colonies when sorbitan monooleate is added to the medium.[10] This smooth phenotype disappears immediately upon subculturing on media without sorbitan monooleate. Similarly, virulent strains of *Mycobacterium tuberculosis* develop into colonies characterized by smooth contour and glistening surface when grown on the surface of agar media containing Tween 80; however, on the surface of media without Tween 80 the same cultures yield much smaller colonies with an extremely ragged outline.[11] It is possible that in these two cases the presence of surface active agents may have modified the restraining effects of the solid medium upon dividing cells (p. 105), thus causing a different arrangement of cells within the colony and consequently a change in the colony's appearance. A third example of temporary modifications of colonial morphology is the change that occurs when genetically smooth cells of *Brucella spp.* are grown in the presence of non-inhibitory concentrations of penicillin.[12] Under such conditions, smooth cells will produce rough or mucoid colonies, the actual phenotype depending upon the strain and the penicillin concentration employed. Subculturing on penicillin-free media or the addition of penicillinase will reverse this effect immediately and will reestablish the smooth phenotype. In this case the biosynthesis of cellular surface components responsible for S characteristics presumably is inhibited in the presence of penicillin.

[10] Rogosa, M. and Mitchell, J. A., J. Bact., *59:*303, 1950.
[11] Dubos, R. J. *et al.*, Am. Rev. Tuberc., *54:*204, 1946.
[12] Braun, W. *et al.*, J. Bact., *64:*41, 1952.

As stated before, changes in colonial morphology can serve as a useful indicator of associated changes in many studies on bacterial variation, particularly in population studies. However, since the colonial morphology is subject to temporary environmental modifications, careful differentiation must be made between changes due to modifications and changes due to mutation. Many reports in the literature are difficult to evaluate because of the failure to recognize and analyze the actual cause of observed colonial variation.

ANTIGENIC MUTANTS

Like colonial characteristics, antigenic or serologic characteristics, especially of pathogens, represent one of the useful taxonomic and diagnostic tools in bacteriology. Therefore, it is not surprising that variation in antigenic characteristics has been known and studied for a long time. Indeed, much of our knowledge regarding the antigenic structure of bacteria has been obtained through studies on the nature of antigenic changes which may occur in bacterial populations *in vitro* as well as *in vivo*. The literature on the manifestations of these changes, dating back almost 50 years, is immense[4, 5] even though the underlying causes of this variability have been either obscure or their interpretation has been contradictory. The studies of the last decade have greatly helped to remove this subject from its obscurity and there is now ample evidence that most changes in antigenic characteristics, like the other characteristics that we have discussed so far, are mutational in origin.

Antigenic types

Adequate descriptions of antigenic characteristics can be found in most textbooks[13] and do not need much elaboration here. There is no uniform nomenclature for the antigenic characteristics of bacteria; the complexity of the different schemes of symbols is matched only by the complexity of the antigenic components themselves. In general, many gram-negative species are characterized by the possession of O (somatic) and H (flagellar) antigens of predominantly protein nature. The O antigens in turn can be subdivided into a number of different components; the H antigens into group and specific antigens. Variation in H antigens is commonly referred to as phase variation. Loss of the O antigen accounts for the S→R

[4] Dubos, R., *The Bacterial Cell*, Harvard Univ. Press, Cambridge, Mass., 1946.

variation, a reduction in O antigens yields antigenically intermediate (I) types, and replacement of the O antigen by a surface antigen with high mucopolysaccharide content results in the so-called M types. Many gram-positive species possess surface antigens of predominantly polysaccharide nature, usually in the form of distinct capsules, which determine their type-specificity. S→R variation in these species is accompanied by the loss of the capsular polysaccharide. In addition, each species can possess specific somatic antigens of protein nature. Since the surface antigen or antigen complex of a cell is responsible for the serological response, a cell may possess additional antigenic characteristics which do not become detectable until the surface antigen is removed either by chemical means or by spontaneous variation affecting the biosynthesis of the specific surface substances. This antigenic multiplicity can be visualized best by comparing the bacterial cell-surface to the layers of an onion where peeling away of one layer will uncover another layer or antigenic surface, a helpful picture which, like most simplifications, does not agree entirely with the facts.

The cause of antigenic changes

We may assume that loss of an antigenic component represents a process similar to that of a biochemical mutation, i.e., a loss of the ability to perform previously possible biosynthetic steps, either by the loss of activity of a specific enzyme or its inability to act at the critical time. Just as an auxotrophic mutant may revert to a prototrophic condition, the loss of, or change in, an antigenic component may be restored by mutation to the original condition, a situation which is amply documented by observations. In fact, it has been possible to demonstrate that in the case of relatively high mutation rates of the H antigen of *Salmonella typhimurium,* mutations from one phase to another and reverse mutations to the original phase may occur frequently enough to permit, during growth in the absence of selective conditions, the establishment of an equilibrium between the two mutants which depends solely on the ratio of the two mutation rates[14] (see Fig. 45).

Phenotypic modifications

Just as an auxotrophic mutant can be made to grow on a minimal medium if the non-synthesized compound is added to the

[14] Stocker, B. A. D., J. Hyg., *47*:398, 1949.

medium, some antigenic mutants may be restored temporarily to their original phenotype if the missing component, or a compound related to it, is supplied exogenously. Two good examples are the ability of R cells of various *Salmonella* species to develop S antigenic characteristics following growth on solid media containing soluble starch[15] or other carbohydrate supplements[16] and the capsule formation by certain *E. coli-mutabile* mutants under the influence of alkaline substances engendered by other mutant cells.[17] In the first case the necessary carbohydrates, normally synthesized by the S cells, presumably were made available to the R cells which had lost the ability to synthesize these carbohydrates. These mutants obviously retained the ability to incorporate such compounds into their cell surface if they were made available to them. Removal of such supplements restored the original R phenotypes. These examples illustrate that antigenic characteristics can be subject to temporary modifications.

Variation in antigenic complexes

Antigenic complexes of the kind encountered in the case of O and H antigens, where antigenic subtypes (e.g., group antigens, specific antigens) can be recognized, frequently show a tendency for simultaneous changes in several or all members of the complex. The studies of Kauffman[15] and of Edwards and Bruner[19] on *Salmonella* extensively illustrate the occurrence of such changes. No decisive genetic interpretation for such phenomena can be offered as yet, but some possible causes can be suggested. The presence or absence of associated changes in antigenic complexes may involve the activity of modifier genes altering the effects of other genes which directly control the biosynthesis of either one or several antigenic components. Another possibility is an interaction among the several genes that may control the individual members of an antigenic complex. This interaction may be of such a nature that changes in one gene can simultaneously interfere with the ac-

[15] Crossley, V. M., Ferguson, M. and Brydson, L., J. Bact., *52*:367, 1946.
[16] Hayakawa, K., Japan J. Exp. Med., *15*:197, 1937.
[17] Zamenhof, J., Heredity, *37*:273, 1946.
[18] Kauffman, F., *The Diagnosis of Salmonella Types*, C. C Thomas, Springfield, Ill., 1950.
[19] Edwards, P. R., Barnes, L. A. and Babcock, M. C., J. Bact., *59*:135, 1950. Bruner, D. W. and Edwards, P. R., J. Bact., *42*:467, 1941; J. Bact., *55*:449, 1948.

tivity of another gene in a manner similar to the interaction between two separate pathways uncovered in the studies on mutations affecting isoleucine synthesis (see p. 98). In other words, genes controlling specific antigenic characteristics may act simultaneously as modifiers for other antigenic characteristics. Associated changes of antigenic components also may be explained on the assumption that the biosynthesis of these components involves common pathways (see Fig. 10). Depending on whether a mutation causes a block in the common biosynthetic pathway (A) or its subsequent side branches (B, C), all or only a few components of an antigenic complex would be affected.

Phase variation in Salmonella

Certain antigenic changes occur spontaneously at very high rates. This is particularly true of changes in flagellar antigen, i.e., phase variation. Stocker[14] recently provided a thorough analysis of mutation rates in phase variation of *Salmonella typhimurium*. These variants were detected by pouring sloppy (0.35%) nutrient agar containing antibody for one phase on the surface of agar plates on which colonies had grown. This resulted in narrow, dense zones of opacity around colonies consisting of cells of the agglutinated phase, whereas colonies of the other phase displayed diffuse zones due to swarming organisms. This method permitted the rapid analysis of the proportion of phase variants that established themselves in liquid cultures originally inoculated with cells of one phase only and maintained, by repeated transfers, in continuous logarithmic growth. Under such conditions, and provided that the growth rates of parent cells and mutant cells are identical, the proportion of mutant cells will increase linearly with the number of generations at a rate proportional to the mutation rate.[20] These prerequisites are fulfilled in the case of the H antigen variation of *S. typhimurium*. Thus Stocker was able to calculate the mutation rates from the slope of increase in proportion of mutant cells (see p. 65 for further details of this method). In nine strains tested, the mutation rates from group to specific phase ranged from 1×10^{-4} to 4.7×10^{-3} per bacterial generation cycle, and the rates of mutation from specific to group phase ranged from 1×10^{-5} to 8.6×10^{-4}. In all cases the mutation rate from group phase to specific phase was higher than the rate of reverse changes. Additional tests involving

[20] Luria, S. E. and Delbrück, M., Genetics, *28*:491, 1943.

replicate cultures and patterned after Luria and Delbrück's fluctuation test (p. 52) substantiated the assumption that the occurrence of phase variants is a spontaneous and undirected event, likely to take place once within approximately 1×10^4 cell divisions, i.e., a mutational change.

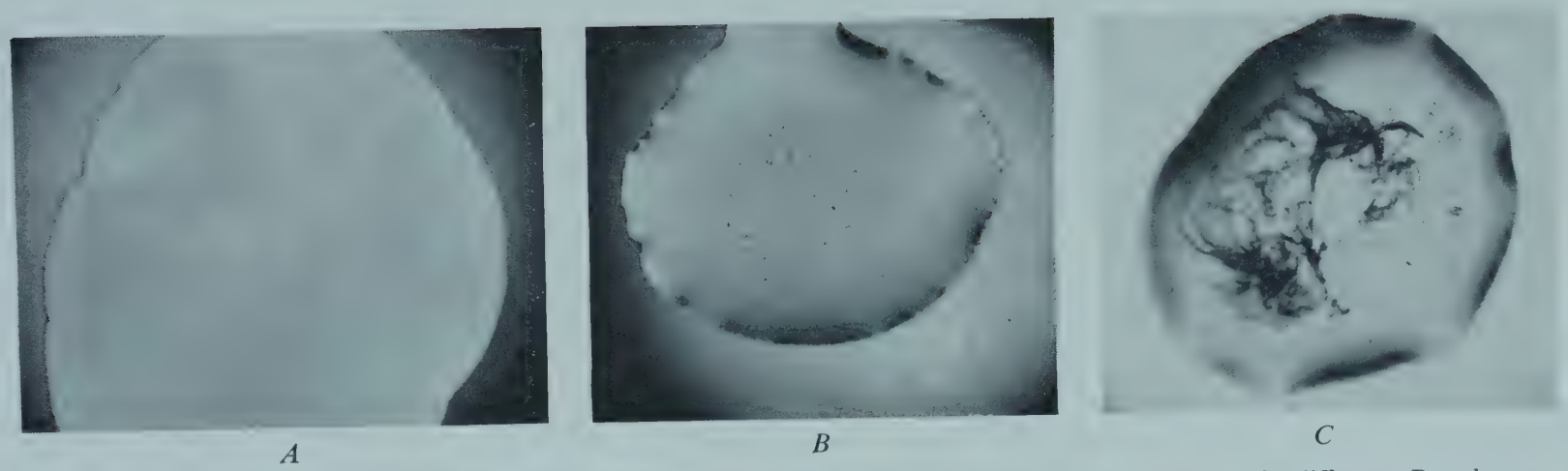
Simple indicators of antigenic changes

The methods of identifying antigenic characteristics are frequently cumbersome. Even under ideal conditions they involve tests on large populations of cells rather than on single cells or their immediate progeny. This undoubtedly has introduced errors into the literature where certain antigenic characteristics have been described as representative for certain cells, whereas they may really represent the overall antigenic characteristics of heterogeneous populations consisting of several antigenically dissimilar cell types. Considerable work is required to identify the antigenic characteristics by serological methods; the *in vivo* testing of associated immunogenic properties is even more time-consuming. For these reasons, correlated characteristics have been useful as rapid indicator of antigenic constitution or changes. One of these is colonial morphology. Even though the terms "smooth" and "rough" were originally applied to colonial morphology,[21] it was soon recognized that changes in colonial morphology were usually associated with changes in antigenic characteristics. Because of the medical importance of the latter, most of the studies on population changes among pathogens were undertaken in an effort to analyze antigenic changes rather than changes in colony morphology. To what extent the use of colonial characteristics as indicators of antigenic characteristics is justified depends on the species under investigation. In some species, changes in these two characteristics are closely linked; in others independent changes in colony morphology and antigenicity are frequent. In the case of several gram-negative species (e.g., *Brucella* species, *B. tularense*, *Salmonella* species, *E. coli*) the acriflavine test has proved to be a far more reliable indicator of antigenic characteristics than mere observation of colony morphology.[22, 23] This simple test consists of suspending cells, preferably from individual colonies, in a drop of saline; a small amount of

[21] De Kruif, P. H., J. Exp. Med., *33*:773, 1921.

[22] Allessandrini, A. and Sabatucci, M., Ann. d'Igiene, *41*:853, 1931.

[23] Braun, W. and Bonestell, A., Amer. J. Vet. Res., *8*:386, 1947.



A *B* *C*

Fig. 41. Reactions occurring after addition of a drop of 1:1000 neutral acriflavine to antigenically different *B. tularense* cells suspended on a slide in a large drop of saline. *A*, Antigenically S cells; *B*, antigenically R cells; *C*, antigenically M cells. Magnified about 20 times.

a 1:1000 solution of neutral acriflavine is then added with the help of an applicator stick. If the cells in the suspension are antigenically smooth they will remain suspended; antigenically rough cells will yield clumps, and antigenically mucoid cells will produce slimy precipitates upon contact with the acriflavine (Fig. 41). Whenever the result of this test differs from the colony appearance, this should be so indicated in the description of the mutant. Thus, the symbol S describes a type that is antigenically and colonially smooth, whereas a smooth colony with R antigenic characteristics, as indicated by a rough acriflavine test, should be labelled S^R. Similarly, S^M would describe a colonially smooth mutant with M antigenic characteristics. Failure to observe such a differentiation between antigenic and colonial characteristics has resulted in considerable confusion in the existing literature since the terms smooth, rough, mucoid, etc., and the symbols S, R, M, etc., often have been applied loosely to either colonial morphology or antigenic characteristics. The suggestion which has been offered by some authors[24] to designate, whenever possible, antigenic characteristics by the symbols S, M, R, etc., and colonial characteristics as smooth, rough, mucoid, etc., appears to be of distinct merit, especially in view of the frequently encountered independent changes between the two characteristics (see p. 133).

Reversions

In the literature on antigenic changes many references can be found to mutational changes in two directions as represented by progressive and reverse changes in antigenic characteristics, e.g., in phase variation or S↔R. Evidence exists which indicates that such changes in two opposite directions do not *necessarily* involve reverse genetic changes but may be due to progressive genetic changes involving a reversal in phenotype only.[5] Thus, genetic changes yielding a phenotypic picture of S to R and back to S ($S \rightleftarrows R$) in *Brucella abortus* were shown to be more correctly represented as changes from S to R to S′ ($S \rightarrow R \rightarrow S'$) where S′ was antigenically similar to S but the mutant S′ differed significantly from the parent S type in the associated characteristic of alanine resistance.[25] Thus,

[24] Topley and Wilson, *Principles of Bacteriology and Immunity*, 3rd Ed., Williams and Wilkins Co., Baltimore, 1946.

[25] Goodlow, R. J., Braun, W. and Mika, L. A., Proc. Soc. Exp. Biol. Med., *76*:786, 1951.

just as in the case of "reverse" mutations from auxotrophy to prototrophy (p. 96) phenotypic reversal is not always indicative of genotypic reversal, i.e., a return of the gene to its original status. A genetic change involving another gene, e.g., a suppressor gene, or a different allelic state of the same gene may cause a phenotype which mimics that of the original parent type.

MUTANTS WITH ALTERED VIRULENCE

Virulence expresses the ability of certain types of bacteria to produce a pathological state in a particular host. It is the end result of interactions between host and parasite, and is governed by certain inherent properties of the bacterium and of the host. Therefore, it is not quite accurate to refer to the virulence of a bacterium *per se* without referring to the host. Nevertheless, such oversimplification is convenient for descriptive purposes, provided it is understood that whenever we talk about properties associated with the virulence of a bacterial cell we are actually referring to properties that affect its relation to the host.

The variability of bacterial properties associated with virulence has been known for a long time and it has been of great basic as well as applied interest to medical bacteriologists.[5, 13] Numerous studies have shown that *in vivo* passage of pathogenic bacteria may often increase their virulence and it is well known that a loss of virulence is likely to occur following prolonged cultivation under laboratory conditions. The evidence accumulated during recent years clearly indicates that these changes involve the establishment of mutants with altered virulence in initially virulent populations (see p. 153). In many instances, the actual nature of the inherent properties which are altered in such mutants is relatively unknown. However, a number of complex properties affecting virulence have been described and they may undergo either simultaneous changes or vary independently of each other.

Inherent properties affecting virulence

Pathogenic bacteria differ from saprophytic bacteria in their ability to invade the tissues of a host and to cause symptoms of disease. These symptoms will ensue unless the invaders are quickly defeated by the host's defense mechanisms, which include the activity of the reticulo-endothelial system. The invasiveness of pathogenic bacteria, therefore, is one of the properties which determine their

virulence, and it depends partly on the manner in which the bacterium can protect itself, e.g., through the possession of a polysaccharide capsule or smooth surface antigen, against the action of the phagocytes. Consequently, changes in the surface structure of bacterial cells, including changes from smooth to non-smooth, usually are associated with simultaneous changes in virulence (see Table 7).

TABLE 7. *Virulence for guinea pigs of several mutants of* Brucella suis *differing in colonial morphology.*

(From *Brucellosis*, a publication of the AAAS, 1950)

Brucella suis TYPE	DOSAGE (ORGANISMS)	INFECTION RATIO (INFECTED / TOTAL)
S = Smooth	14	9/9
	140	9/9
I_1 = Intermediate$_1$	12	4/8
	120	10/10
	1200	8/8
I_2 = Intermediate$_2$	10	0/10
	100	1/10
	1000	2/9
I_3 = Intermediate$_3$	8.2	0/10
	82	0/4
	820	6/10
R = Rough	8.6	0/8
	86	0/6
	860	5/9
M = Mucoid	9.4	0/9
	94	1/8
	940	2/7

Another property that contributes to the virulence of many pathogenic strains is the production of toxins. This property is subject to variation and its loss can cause a loss of virulence in toxigenic species. Many other inherent factors seem to contribute to the virulence of bacteria. Some of them are poorly understood; others have been described as associated with the production of hemolysins, leukocidins, coagulase, fibrinolysins, or hyaluronidase.[26, 27] It can be hoped that all of these properties will ultimately be describable in biochemical terms; changes in these properties

[26] See Burrows, W., *Textbook of Bacteriology*, 15th Ed., W. B. Saunders Co., Philadelphia, 1949.

[27] Mutants differing in hyaluronidase production have been observed in studies with hemolytic streptococci by Sallman, B., J. Bact., 62:741, 1951.

can then be analyzed and recognized just as other phenomena involving biochemical mutants.

Associated changes

So far most studies on changes in virulence which can be subjected to genetic analysis have utilized easily identifiable associated changes, for example, altered antigenicity or colonial morphology, as indicators. This has been necessary because of the cumbersome techniques associated with titrations of virulence in animals or plants and seems justified for species in which there is a high degree of association between these characteristics. Such studies[5] have revealed that changes in virulence occur spontaneously and without a directing influence of the environment, just as in the case of changes in colonial morphology and antigenicity. They manifest themselves initially only in a few cells of a population consisting predominantly of cells with different virulence. Therefore, changes in virulence behave as mutational changes, and much of the information reviewed in the preceding sections on antigenic and colonial mutants also applies to virulence characteristics. In fact, the usual close association between virulence and colonial morphology has permitted the isolation of mutants with altered virulence on the basis of differences in colonial appearance. For example, a systematic isolation of clones differing in virulence was easily accomplished in *Bacterium tularense* when it was found that differences in colonial morphology could be detected with the help of the oblique lighting system.[28] Cultures which in animal titrations had revealed unequal virulence were found to consist of mixtures of cells with different virulence which could be separated on the basis of colonial morphology (Fig. 38). Most of the resulting clones displayed considerable stability in virulence and colonial characteristics. Similarly, clones with differences in virulence have been isolated on the basis of colonial morphology in a number of bacterial species. An additional interesting example is provided by studies with *Salmonella typhimurium,*[29] where clear-cut differences in colonial morphology became apparent when mixed cultures, streaked on MacConkey's agar, were viewed with oblique lighting. Two colonial types (Fig. 39), differing significantly in virulence but not in their acriflavine reactions or antigenic formulae, were recognized and isolated. This

[28] Eigelsbach, H. T., Braun, W. and Herring, R. D., J. Bact., *61*:557, 1951.
[29] Page, L. A., Goodlow, R. J. and Braun, W., J. Bact., *62*:639, 1951.

also represents one example of the occasional occurrence of associated changes in virulence and colonial morphology without changes in antigenic characteristics.

SOME GENETIC PROBLEMS COMMON TO COLONIAL, ANTIGENIC AND VIRULENCE MUTANTS

The foregoing descriptions have illustrated that in many instances the available information pertaining to colonial mutants is also applicable to phenomena associated with mutants differing in antigenicity or virulence, and *vice versa*. In the case of some genetic problems associated with these mutants the extent of such overlapping information is particularly pronounced; problems belonging in this category will be considered now.

Mutation rates

Evidence for the mutational origin of spontaneous variants differing in colonial morphology, antigenicity, and virulence is convincing[5] but direct proof and reliable data on mutation rates as supplied by fluctuation tests are scarce. It will be recalled that the principal feature of the fluctuation test is the ability to recognize small numbers of mutants among an overwhelming number of parent cells by the specific elimination of the non-mutant background. For example, streptomycin-resistant mutants can be separated easily by the lack of growth of streptomycin-susceptible parent cells in the presence of the antibiotic. Such specifically selective agents, capable of eliminating all cells of one type in favor of variant cells, are relatively unknown as far as colonial morphology, antigenicity and virulence are concerned. So far, there is only one case on record where selective agents *and* the fluctuation test have been employed for the estimation of mutation rates involving antigenic and colonial changes from S→R. In studies with *Brucella* species it had been found that S cells are far more susceptible to the growth-inhibitory effect of D-alanine or salicylate than R cells (p. 153). Therefore, soft (0.75%) agar media, containing D-alanine or salicylate, could be used as screening media on which S cells developed into microcolonies only, whereas R cells developed into colonies of normal size (Fig. 42). With the help of such screening media and the fluctuation test (Fig. 22) a spontaneous mutation rate of 1×10^{-7} was obtained for S→R changes in a strain of *Brucella abortus*.[30] It also

[30] Braun, W. and Ciaccio, E., Bact. Proc., p. 41, 1952.

has been reported that S and R variants of intestinal pathogens display differential susceptibility to desoxycholate,[31] but so far this effect has not been exploited for fluctuation tests.

In the absence of effective means for eliminating the predominant parent cells, visual inspection of easily recognized differences between mutants and the parent type has been used in a few cases to gain information on mutation rates. Reference already has been

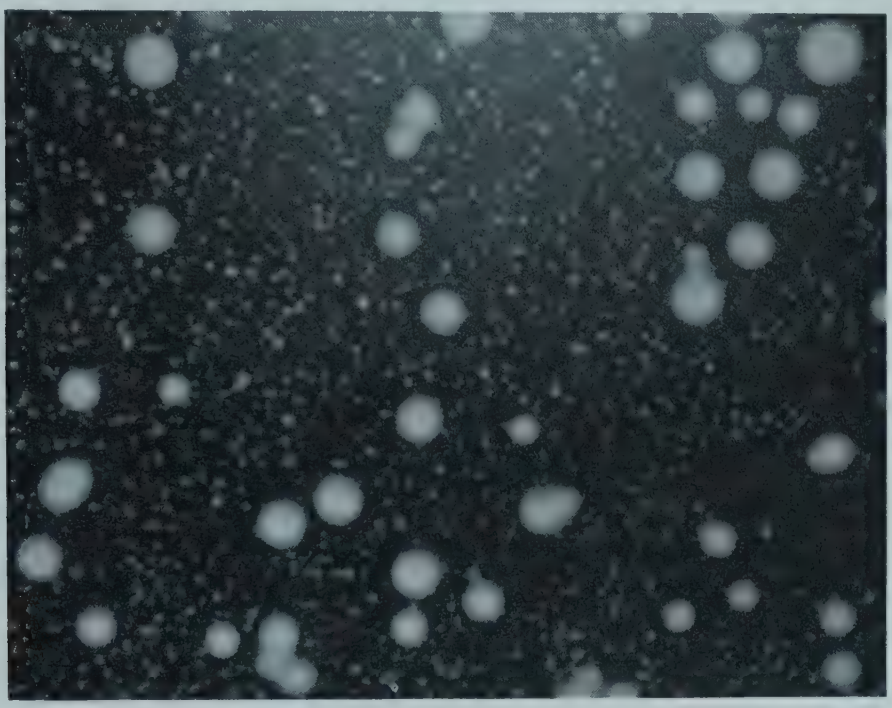


Fig. 42. Colonies which developed from S and R cells of *Brucella abortus* on soft (0.75 per cent) agar containing 3.0 mg/ml of DL-alanine. Tiny colonies developed from S cells, large colonies from R cells. Magnified about 15 times.

made to mutation rates of H antigens of *Salmonella typhimurium* (p. 117) where the mutants were detected by pouring samples of liquid cultures into sloppy agar containing antibody for one phase. In another study with *S. typhimurium* the approximate mutation rate from S^t to S^o, involving simultaneous changes in colonial morphology and virulence, was estimated with the help of the fluctuation test and visual inspection of colonies.[29] The rate was found to be 3×10^{-5} per fission. Lincoln[32] employed the fluctuation test and visual inspection of colonies to estimate spontaneous mutation

[31] Brodie, J. and Shepherd, W., J. Gen. Microbiol., *3*:74, 1949.
[32] Lincoln, R. E., J. Bact., *54*:745, 1947.

rates involving changes in colonial morphology (various rough, smooth, and small colony types) of *Bacterium stewartii (Phytomonas stewartii)*. Depending on the temperature and strain employed, rates varying from 2×10^{-4} to 3×10^{-7} were observed. A more direct approach was used by Zelle[33] who calculated the mutation rate for S→R changes in an unstable strain of *S. typhimurium* by separating cells *in vitro* as they divided. He observed that one R cell arose during 148 divisions of S cells.

From such data it becomes evident that mutations affecting colonial morphology can occur at greatly different rates, ranging from 1×10^{-2} to 1×10^{-10} per fission cycle. Changes in colonial morphology occurring in unstable strains, such as the *S. typhimurium* strain tested by Zelle, are representative of mutations occurring at extremely high rates; at the other extreme are the low rates of mutation observed for colonial characteristics that are associated with growth-requirements or antibiotic resistance (p. 110). The rates of mutation affecting antigenic and virulence characteristics presumably may vary over a similarly wide range, depending partly on the species, the strain and the actual cellular component involved. This conclusion appears justified on the basis of (1) the frequent correlation among changes in colonial morphology, antigenicity and virulence, (2) the limited evidence available from fluctuation tests, and (3) the many indicative observations on the variable stability of these characteristics during prolonged cultivation. However, it must be kept in mind that the latter evidence, i.e., cultural stability, is not necessarily conclusive since in observations based upon population changes it is often difficult to separate the effects of selection from those of mutation. In addition, mutants affecting antigenicity and or virulence are often difficult to recognize; many of them may remain undetected unless environmental conditions permit their establishment within the parent population. These problems will be discussed in more detail in Chapter 8.

Nuclear or cytoplasmic factors

At the present time it is a matter of conjecture whether all of these changes represent changes in nuclear factors (genes) or whether some may involve changes in cytoplasmic factors. A spontaneous change in a hereditary characteristic does not necessarily indicate a change in a nuclear factor. In Chapter 1 reference has

[33] Zelle, M., J. Inf. Dis., *71*:131, 1942.

been made to the fact that characteristics under the control of cytoplasmic factors are equally apt to change spontaneously. However, sexual reproduction, permitting an analysis of the distribution of characteristics in the progeny from appropriate matings, is a prerequisite for detecting the involvement of cytoplasmic factors (p. 20). In the absence of such test systems among most bacteria (see Chapters 11 and 12 for certain exceptions), no definite proof for the existence of cytoplasmic factors controlling hereditary characteristics is available so far. Nevertheless, Ephrussi's recent data on yeast (p. 25), showing that cytoplasmic factors can be responsible for the control of certain colonial characteristics, may serve as a model for mechanisms that might occur in bacteria. Similarly, Sonneborn's findings on the cytoplasmic control of antigenic changes in *Paramecium* (p. 23) could provide an example of phenomena that might play a role in antigenic variation of bacteria. Yet it must be remembered that both in the case of cytoplasmic inheritance of colonial morphology in yeast as well as in antigenic transformation of *Paramecium,* relatively non-specific environmental conditions (acriflavine, antisera, temperature, altered nutrition, etc.) are capable of inducing fairly stable changes of the characteristics involved. Similar inductive effects of non-specific environmental conditions have not been observed in bacterial populations thus far.[34] Even though antisera, nutrition, and temperature can cause changes in colonial morphology, antigenicity or virulence of bacterial populations, all such changes so far can be explained on the basis of undirected, spontaneous nuclear changes in one or a few cells and the subsequent selection of such mutants (see Chapter 8). Therefore, in the absence of evidence to the contrary, and in view of the fact that the changes discussed in this chapter arise spontaneously and are maintained in the absence of specific environmental conditions, it appears reasonable to regard them as due to mutations that are analogous to those described for nutritional requirements and resistance to inhibitory agents.

Antigenicity and virulence of auxotrophic mutants

Investigations on the actual nature of the biosynthetic steps involved in the development of antigenic and virulence characteristics, utilizing the type of method that has been so fruitful in the

[34] However, see Chapter 12 for bacterial transformations in the presence of specific environmental agents.

study of nutritional mutants, are just beginning. This method consists of testing auxotrophic mutants of species with well analyzed antigenic and virulence properties for the retention or loss of such properties. Studies of this type have been carried out with *Salmonella typhimurium*,[35, 36] *S. typhosa*,[37] and *Klebsiella pneumoniae*.[38] In the studies with *S. typhimurium*,[35] no significant alterations of serological characteristics were found among the 79 different radiation-induced auxotrophic mutants which were isolated with the help of the penicillin screening technique. However, several mutants displayed altered immunogenic properties, e.g., adenine-, hydroxyproline-, and arginine-requiring mutants displayed a loss of virulence and immunogenicity in mice; in contrast, leucine- and serine-requiring mutants proved to be low in virulence but high in their capacity to immunize (Fig. 43). Interestingly enough, different, independently isolated, histidine-requiring mutants showed dissimilar immunizing capacity. It is difficult to assess from the available data whether such findings indicate the existence of a simultaneous genetic block interfering with the synthesis of the essential growth factors as well as with the synthesis of the immunogenic cell components. An alternate possibility is the failure of such auxotrophic mutants to propagate as fast *in vivo* as their prototrophic, immunogenic parent cells. Thus a decrease in the rate of multiplication *in vivo* might be a contributory factor for the decreased immunogenic capacities of some of these mutants. These studies also revealed that most auxotrophs displayed altered virulence in addition to their altered nutritional requirements. Thus, threonine-, phenylalanine-, or methionine-requiring mutants showed increased virulence, while adenine-, hydroxyproline-, arginine-, isoleucine- and leucine-requiring mutants displayed less virulence than the prototrophic parent cells. As in the case of immunogenicity, independently isolated mutants with the same requirement did not always show identical virulence, e.g., different histidine-requiring mutants had different virulence. In a more recent publica-

[35] Gowen, J. W., Stadler, J., Plough, H. H. and Miller, H. N., Genetics, *36*: 553, 1951.

[36] Gowen, J. W., Stadler, J., Plough, H. H. and Miller, H. N., Genetics, *in press*.

[37] Bacon, G. A., Burrows, T. W. and Yates, M., Brit. J. Exp. Path., *31*:714, 1950; *32*:85, 1951.

[38] Garber, E. D., Hackett, A. J. and Franklin, R., Proc. Nat. Acad. Sci., *38*: 693, 1952.

tion[36] the virulence of auxotrophic mutants isolated from three different strains of *S. typhimurium* was reported. One of the prototrophic parent strains (W-4) possessed a high level of virulence when tested in susceptible mice; the second (W-3), an intermediate level, and the third (W-2), a low level. Auxotrophic mutants isolated from the first two strains tended to display greatly decreased virulence, and mutants isolated from the parent strain with low virulence tended to show similar or increased virulence. Figure 43 indicates such trends and in addition illustrates the changes in immunogenic capacity. The abscissa of this distribution diagram indicates the virulence of the auxotrophic mutants; the ordinate indicates their immunogenic capacity. The solid line in this diagram indicates that there is a high positive curvilinear correlation between the virulence of a mutant and its immunogenic capacity. Notable exceptions to such correlations are such mutants as adenine-79, which, though avirulent, confer 100 per cent protection against a challenge with a strain which kills all mice not previously exposed to *S. typhimurium* cells. Figure 43 also illustrates a point previously mentioned, namely, that independently isolated mutants with identical growth requirements, e.g., adenine-requiring mutants 76, 78, 79, 80 and 85, can display dissimilar virulence and immunogenicity. Gowen et al.[36] concluded from this finding "that the particular chemical changes observed in the mutants may not be the primary causes of the variations observed in either virulence or immunizing capacity. The observed variations could be due to mutations of other genes arising simultaneously with the particular metabolite mutant, or the genes controlling a given metabolite requirement are found at several loci and these genes have multiple and different effects,—the variation of any one of which would give the metabolite requirement but a virulence or immunity different from that of another gene."

More specific information on the reasons for the loss of virulence in certain auxotrophs was obtained in the studies on auxotrophic mutants of *S. typhosa*.[37] By exposure to x-ray, ultraviolet irradiation or nitrogen mustard, and subsequent screening with the help of the penicillin technique, Bacon et al. isolated a total of 93 auxotrophic mutants representing 25 different types. Tests in mice revealed that the majority of mutants had retained full virulence. Among those which showed changes in virulence, the most notable loss of virulence was associated with mutants deficient in their

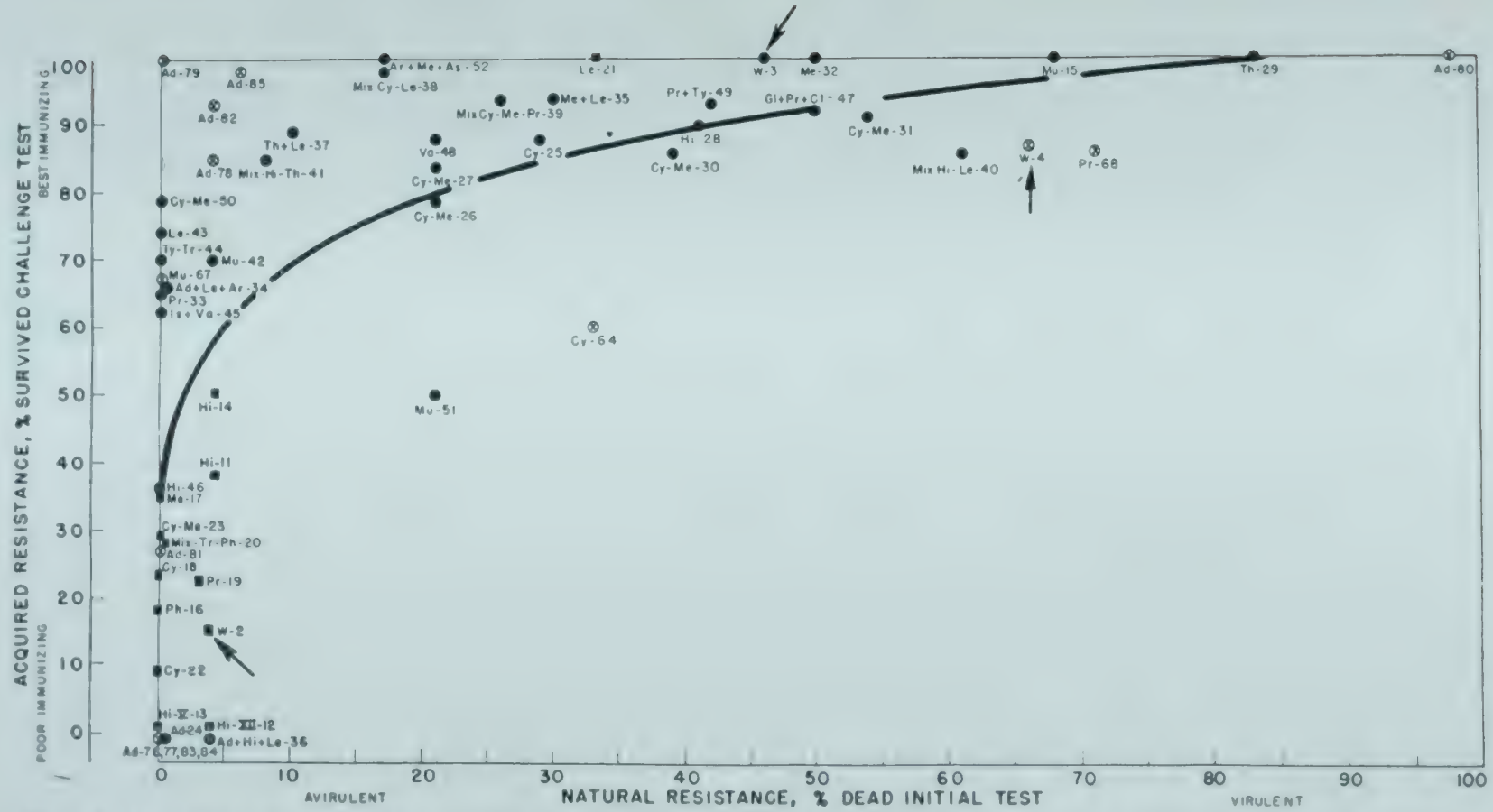


Fig. 43. Virulence and immunizing capacity of various auxotrophic mutants of *Salmonella typhimurium*. The abscissa represents virulence, the ordinate immunizing capacity. *W-2* is the avirulent parent strain and all mutants originating from this strain are designated as ■; *W-3* is a parent strain of intermediate virulence, mutants originating from this strain are indicated by ●; *W-4* is a parent strain of relatively high virulence, mutants derived from it are shown as ⊗. The specific requirements of the mutants are indicated by the first two letters of the following amino acids: adenine, arginine, cysteine, glutamic acid, histidine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. Alternative requirements are indicated by "Mix" and are probably due to a mixture of distinct types; requirement for more than one compound is indicated by "+". (From Gowen, Stadler, Plough and Miller.[7])

ability to synthesize purines, para-aminobenzoic acid (PAB), or aspartic acid, and with certain cystine- (1 of 11) or histidine-requiring (2 of 10) mutants. It is noteworthy that despite the significant loss of virulence of purine-requiring mutants such mutants conformed in colonial characteristics, antigenic structure and Vi-phage sensitivity to the accepted criteria defining virulent *S. typhosa* strains. The authors have suggested that the observed loss of virulence of certain mutants requiring specific growth factors could be due to at least three possible mechanisms: (1) the required growth factor may participate in the synthesis of specific constituents of the cell involved in virulence and the mutant cells may receive enough of this factor from exogenous sources in the host to permit their multiplication, but not enough to permit the same level of synthesis of the virulence controlling components; (2) the growth factor itself may be one moiety of a non-vital surface complex important for the manifestation of virulence, and its supply may be limited in mutant cells which have to incorporate it from exogenous sources instead of synthesizing it; and (3) the limited availability of the required growth factor in the host may prevent the multiplication of the inoculated mutant cells to toxic levels. Experiments with mice, utilizing the less virulent purine-, PAB-, or aspartic acid-requiring mutants, revealed that the last mentioned mechanism was responsible for the lowered virulence of the auxotrophs thus far analyzed. The auxotrophs proved to multiply far more slowly in the host than the prototrophic parent cells and it was demonstrated that this decreased multiplication rate was due to a limited availability of the specific growth factors in the mouse. In contrast, mice were found to contain ample levels of growth factors required for those auxotrophic mutants which failed to show a loss of virulence. As expected, full virulence of the purine-, PAB-, and aspartic acid-requiring mutants was restored in mutant-infected mice by the injection or, in some cases, by the feeding of the specific growth factor. Evidence was obtained indicating that the seasonal variation which had been observed in the virulence of these mutants was due to seasonal changes in the availability of the specific nutrilites in the host. Also, dissimilar mutants of low virulence exerted a synergistic effect when mixed suspensions of such mutants with different growth factor requirements were injected into mice (Table 8). This increased virulence probably resulted from the mutual feeding of the mutants, i.e., mutant A supported the growth of mutant B by excreting the

specific growth factor required by B. and vice versa. Such mutual feeding was also observed when mixed suspensions of these mutants were grown *in vitro*. It may well be that these interesting findings on seasonal variations and synergistic effects of auxotrophs on virulence reflect a mechanism for changes in virulence that can occur in natural infections. The generality of these findings is indicated by the observation that similar mechanisms can occur in other pathogens. Purine-requiring auxotrophs of *K. pneumoniae* also displayed a loss of virulence. As in the case of *S. typhosa*, full viru-

TABLE 8. *The synergistic effect of purine-requiring and PAB-requiring mutants of* Salmonella typhosa *tested in mice.*

(From Bacon, Burrows, and Yates[37])

TOTAL DOSE OF ORGANISMS	DEATHS OUT OF 20 MICE INJECTED WITH		
	purine-requiring mutant #479	PAB-requiring mutant #20063	#479 + #20063
1×10^8	0	0	2
2×10^8	0	1	17
4×10^8	2	1	18
8×10^8	9	10	20

lence was restored when purine was administered to mice infected with such mutants.[38]

In the studies with *S. typhosa* described above, some interesting observations were made also in regard to antigenic characteristics. It was found that auxotrophic mutants with lowered virulence retained the Vi antigen. Also, no consistent correlation between behavior towards O antiserum and virulence was detectable in these mutants, an observation which is of particular interest since the O resistance of naturally occurring Vi + O strains has been regarded as an indicator of virulence. A reduction of Vi + O antigens was detected by serological tests in a histidine-requiring mutant, a marked O antigen deficiency was found in another histidine-requiring mutant, and a reduction of Vi antigen was noted in a cystine-requiring strain. In addition to the change in antigenic properties, these last three mutants were morphologically altered and were of lower virulence.

It can be noted that these studies thus far have failed to uncover any causal relationship between specific metabolic blocks and the

production of components associated with antigenic or virulence characteristics. However, it may be anticipated that such relationships may be uncovered in future studies which will then permit the analysis of biosynthetic pathways involved in the development of these characteristics.

Before concluding this section, reference must be made to the already briefly mentioned relationship between changes in resistance to certain amino acids and changes in colonial, antigenic and virulence characteristics recently observed in studies with *Brucella* species.[25, 39] It was found that S, R, and M mutants of these species may differ significantly in their resistance to the inhibitory effects of D-alanine and DL-valine. These amino acids normally accumulate in cultures of these organisms as end products of their metabolism (see also pp. 152–156).

INDEPENDENT AND ASSOCIATED CHANGES IN COLONIAL, ANTIGENIC, VIRULENCE AND OTHER CHARACTERISTICS

Throughout this chapter repeated reference has been made to the frequent occurrence of simultaneous changes in colonial morphology, antigenicity and virulence. There are still other characteristics which are apt to change in association with the above named characteristics, e.g., cell morphology, stability in saline, sensitivity to bacteriophage, biochemical activities, antibiotic resistance, and ability to sporulate.[40] The occurrence of such associated changes attained considerable significance in the discussions that raged at one time in regard to the nature of changes involving colonial, virulence, antigenic and other associated characteristics. As has been pointed out in Chapter 2, De Kruif, Mellon, Hadley and others regarded such changes as part of a regular life-cycle. The demonstration of the fairly frequent occurrence of independent changes in these characteristics served as important evidence against the concept of regular life-cycles in the days preceding the introduction of genetic techniques into studies with bacteria.[40] Table 9 shows some representative examples of the independent changes that have been observed in studies on the characteristics mentioned above. In inspecting this table it should be kept in mind that the characteristics cited in the first column are identical with those

[39] Goodlow, R. J., Tucker, L., Braun, W. and Mika, L. A., J. Bact., *63*:681, 1952.

[40] See Braun, W., Bact. Rev., *11*:75, 1947.

TABLE 9. *Examples of independent changes among characteristics that frequently show associated changes.*

CHARACTERISTICS INVOLVED	SPECIES	REFERENCE
Cell morphology, instability in saline, inagglutinability in anti-sera, and colonial morphology	*Klebsiella pneumoniae*	Yale J. Biol. Med., *16*:629, 1944
Cell morphology, colonial morphology, production of somatic antigen, and pigment production	*Serratia marcescens*	J. Bact., *34*:255, 1937
	Gaffkya tetragena	J. Bact., *31*:407, 1936
Cell morphology and colonial morphology	*Bacillus megatherium*	J. Bact., *38*:41, 1939
Colonial morphology and capsular polysaccharide production	*Streptococcus sp.*	J. Inf. Dis., *79*:148, 1946
Colonial morphology and fermentative abilities	*Shigella sonnei*	J. Bact., *31*:309, 1936
	Bacillus anthracis	J. Bact., *25*:49, 1933
Colonial morphology, antigenicity, and fermentative abilities	*Escherichia coli*	J. Bact., *31*:453, 1936
Colonial morphology, carbohydrate fermentation, toxin production, and virulence	*Streptococcus sp.*	J. Bact., *32*:105, 1936
Colonial morphology and virulence	*Corynebacterium diphtheriae*	J. Bact., *25*:97, 1933
	Pasteurella pestis	J. Inf. Dis., *73*:124, 1943
	M. tuberculosis	Quart. Bull. Sea View Hosp., *7*:400, 1942
Colonial morphology and antigenicity	*Noguchia granulosis*	J. Inf. Dis., *59*:244, 1936
	Brucella abortus	Amer. J. Vet. Res., *8*:386, 1947
	Salmonella typhosa	J. Hyg., *35*:428, 1935
Virulence and polysaccharide antigens	*Salmonella typhimurium*	J. Bact., *40*:197, 1940
Virulence, agglutinability, growth requirements, hemagglutination, alum precipitability, and protective antigenicity	*Hemophilus pertussis*	J. Gen. Microbiol., *5*:531, 1951
Toxigenicity and colonial morphology	*Clostridium botulinum*	Mikrobiol. Zhur., *7*:63, 1940
	Shigella dysenteriae	Compt. rend. Soc. biol., *136*:478, 1942
Phage sensitivity and colonial morphology	*Streptococcus sp.*	Arch. inst. biol. (São Paulo), *6*:121, 1935

which frequently show associated changes in the same species. The question still remains why the various characteristics discussed in this chapter may show either independent or associated changes. In an attempt to answer this question it must be stated first that a great number of constantly associated changes undoubtedly can be attributed to the occurrence of one basic change. For example, merely the loss of capsular polysaccharide can account for several of the usual characteristics of the unencapsulated R mutants, such as (1) loss of type-specific antigenicity; (2) increased agglutinability by salt through increase of lipid instead of polysaccharide components at the cell surface; (3) altered sensitivity to bacteriophage, and, with some notable exceptions, (4) a loss in virulence[13] (*Bacillus anthracis* and the hemolytic streptococci are examples of the well-known exceptions). This situation appears similar to the previously mentioned correlation between ultraviolet-resistance, nitrogen mustard-resistance and H_2O_2-resistance in certain *E. coli* mutants which seems to reflect the occurrence of only one basic change, namely general resistance to oxidizing influences. However, changes such as those cited in Table 9 must be of a different nature because in these cases a group of characteristics may undergo either associated or independent changes. As in the case of variation in antigenic complexes (p. 116), several different mechanisms may be postulated for such phenomena. First, it is possible to assign the cause to a single mutational change affecting either one of different levels of gene-controlled processes, a scheme that has been useful in the interpretation of genetic studies on higher organisms and that has also been applied to explain the metabolic changes which may or may not occur simultaneously with changes in phage-resistance of *E. coli*.[41] Figure 44 illustrates the manner in which this interpretation can be applied to the occurrence of associated or independent changes in an arbitrarily chosen set of characteristics involving colony morphology, cell morphology, stability in saline, and virulence. It can be assumed that a common precursor, *a*, exists for the biosynthetic processes leading to the development of these characteristics. Mutation A affecting process *a*, and thereby all subsequent processes, will cause simultaneous changes in all four characteristics; another mutation, B, may affect process *b* only and thus cause a change in virulence without changing colony morphology, cell morphology, and stability in saline; at another

[41] Anderson, E. H., Proc. Nat. Acad. Sci., *30*:397, 1944.

time, a mutation C, which affects process *c*, may occur and cause changes in colony morphology, cell morphology, and stability in saline without altering virulence; and so on. The bacteriological literature contains many examples supporting this type of interpretation.[40] Another mechanism that may be responsible for the occasional occurrence of associated changes was first proposed by Luria[42] following studies on mutations affecting resistance to phage

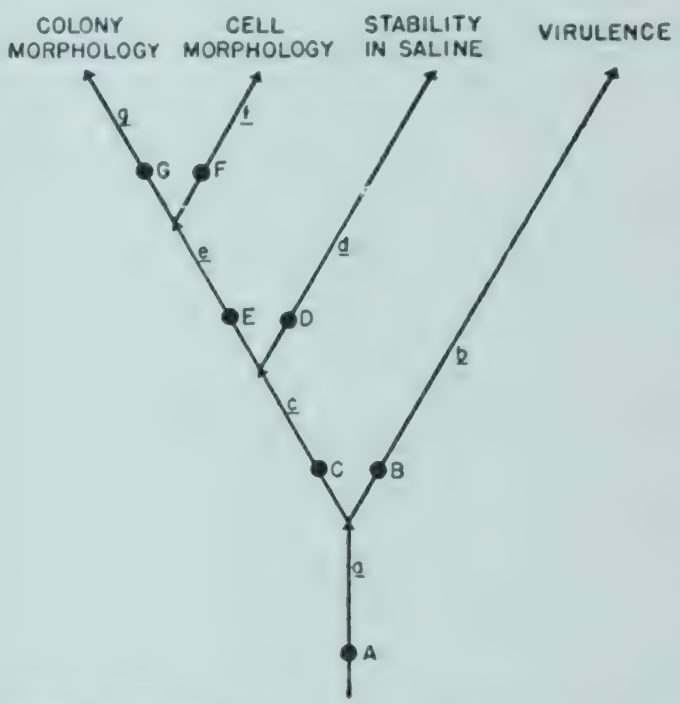


Fig. 44. Diagram illustrating a mechanism by which the occurrence of linked and non-linked variation of characteristics may be caused. The order of characteristics in the above diagram has been chosen arbitrarily; for further explanation see text. (From Braun.[40])

in *E. coli*. His results indicated that the wild type allele of one gene may act as a suppressor for manifestations of the effects of the mutant allele of another gene. For example, a product of the biosynthetic steps controlled by gene X might block processes controlled by gene Y, but when gene X mutates to X′ this block would disappear and gene Y could now manifest its effect. We can refer again to the valine-isoleucine inhibition uncovered in studies with *Neurospora* and *E. coli* (p. 98), as a beautiful example of the feasibility of such a concept. It will be remembered that in this case a mutation affecting the biosynthesis of isoleucine caused an

[42] Luria, S. E., Genetics, *30*:84, 1945.

accumulation of an intermediate product prior to the blocked synthetic step, and this accumulated product in turn caused an inhibition of an entirely unrelated biosynthetic pathway. Finally, still another mechanism that might be responsible for simultaneous changes in unrelated characteristics would be an influence of one mutation on the rate of occurrence of another mutation. Such a mechanism, though often suspected, still lacks substantial proof.

CHAPTER 8

Population Changes Due to Mutation and Selection: I

So far we have considered primarily changes which occur in the genetic make-up of individual cells. We have seen how such changes can be detected most conveniently when environmental conditions are adjusted in such a manner that only the progeny of these altered cells and none of the parent cells can survive. Although such specifically selective conditions do occur occasionally in nature, e.g., after exposure of pathogens to antibiotics, they do not represent the more common condition where cells with different genotypes compete within a population. Since specific mutants may arise at an "average" rate of 1 in 10^9 cells and since most bacterial populations reach such a population density very rapidly, it is evident that after a brief period of growth practically all bacterial populations, even if started from a single cell, contain one or more mutant cells. If a mutant cell is better adapted to the existent environmental conditions than the parent cells, i.e., if it can survive better and propagate faster, its progeny will constitute an ever increasing proportion of the total population and the genotypic and phenotypic complexion of the population will change. Thus, in dealing with bacteria, population changes are likely even if clones are used as starting material, i.e., if the inoculum of a culture consists of a *homogeneous* population, containing only genetically identical cells. This situation becomes even more complex if the inoculum constitutes a *heterogeneous* population, i.e., contains cells with different genotype, a situation which is most frequently encountered in bacteriological work. In the case of initially heterogeneous cultures, immediate selective growth of certain, already present, mutants may occur in addition to the selective establishment of mutants arising following growth of the culture. Therefore, population changes are even more likely to occur, and occur more rapidly, if heterogeneous rather than

homogeneous inocula are used. In microorganisms, population changes represent an ever-present problem. The recognition of population changes and an understanding of population dynamics may be considered prerequisite to any study with bacteria.

In dealing with bacterial population changes due to mutation one has to consider at least two types of representative conditions: (1) changes occurring under relatively constant environmental conditions, and (2) changes occurring when the environment continuously changes, as the result of natural or artificial exposure to new cultural conditions or as the result of alteration in cultural conditions produced by the metabolic activity of the bacteria themselves.

Population Changes during Continued Growth under Relatively Constant Environmental Conditions

Population equilibria between mutants of equal growth rate

Constant environmental conditions may occasionally prevail for relatively long periods in nature but they are rarely found under normal laboratory conditions. An approximation of such conditions can be achieved if cultures are frequently transferred to fresh media, e.g., every day. Constant environmental conditions can be achieved more successfully if a continuous culture apparatus such as the chemostat (see p. 67), permitting logarithmic growth indefinitely, is used. If mutants arise within a population growing under such constant conditions, and if such mutants have the same growth rate as the parent cells, then the rate of establishment of such mutants will be proportional to the mutation rate. Actually it will diminish with time since the percentage of the original cell type susceptible to mutation will gradually decrease. *Mutation pressure* is a term applied to the cause of population changes under conditions where the continued though infrequent occurrence of mutants alone is responsible for changes in the population and where there is no specific selective advantage for either the parent cells or the mutant cells since both grow at equal rates. In a preceding chapter we have referred to the fact that back-mutations are apt to occur. Such back-mutations, occurring after the proportion of mutant cells is sufficiently large to yield mutant cells of their own, will further reduce the increase of mutants in a growing population. This will ultimately cause the attainment of an equilibrium level between the proportion of mutant type to parent type.

This was pointed out by Bunting[1] in her studies on color mutants of *Serratia marcescens*. She showed that at the time of equilibrium the ratio of the two cell types will equal the ratio of their respective mutation rates. Thus, in populations where cells undergo mutation at the rate m, and back-mutation at the rate b,

$$\frac{\text{number of cells of parent type}}{\text{number of cells of mutant type}} = \frac{b}{m}$$

at time of equilibrium.

This will hold true only if the growth rates of parent and mutant types are equal, a situation which is infrequently met. Fairly high

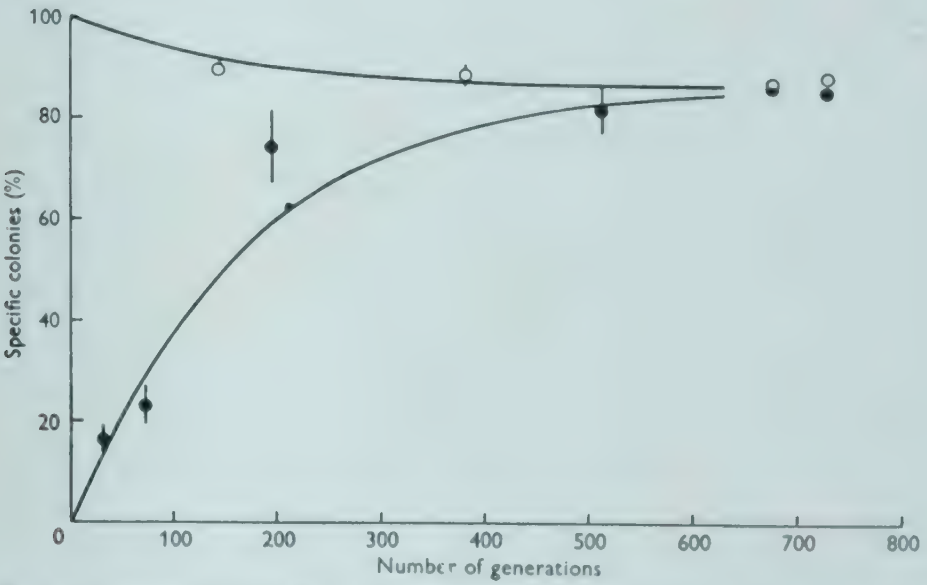


Fig. 45. Attainment of an equilibrium state between group and specific phase mutants of *S. typhimurium* after prolonged logarithmic growth. Observations on mutation from group to specific phase are represented by solid circles, observations on mutation from specific to group phase by open circles. Lines correspond to mutation rates of 5.2×10^{-3} and 8.8×10^{-4}. (From Stocker.[2])

mutation rates are also required for the observation of this phenomenon, because more than 400 generations are required for the attainment of this population equilibrium if the mutation rates are as high as 1×10^{-4}. Nevertheless, Stocker,[2] in his studies on rates of mutation of antigenic phase in *Salmonella typhimurium* (see p.

[1] Bunting, M. I., Cold Spring Harbor Symp. Quant. Biol., *11*:25, 1946.
[2] Stocker, B. A. D., J. Hyg., *47*:398, 1949.

117), was able to provide an additional experimental demonstration for such population equilibria, controlled by the ratio between "forward" and "back" mutation rates. For example, under conditions of continuous logarithmic growth one strain yielded after 600 generations about 14 per cent group-phase cells and 86 per cent specific-phase cells, regardless of whether the initial culture contained specific or group cells only (Fig. 45). This is in accordance with the theoretically calculated expectation of an exponential approach towards an equilibrium state involving cells with a mutation rate of 5.2×10^{-3} for the change from group to specific phase and a rate of 8.8×10^{-4} for the reverse change. The actual mutation rates found were in close agreement with those postulated; they turned out to be 4.7×10^{-3} and 8.6×10^{-4}, respectively.

"Periodic selection"

In some of these population studies Stocker noticed an anomalous decrease in the proportion of mutant cells after about the 500th

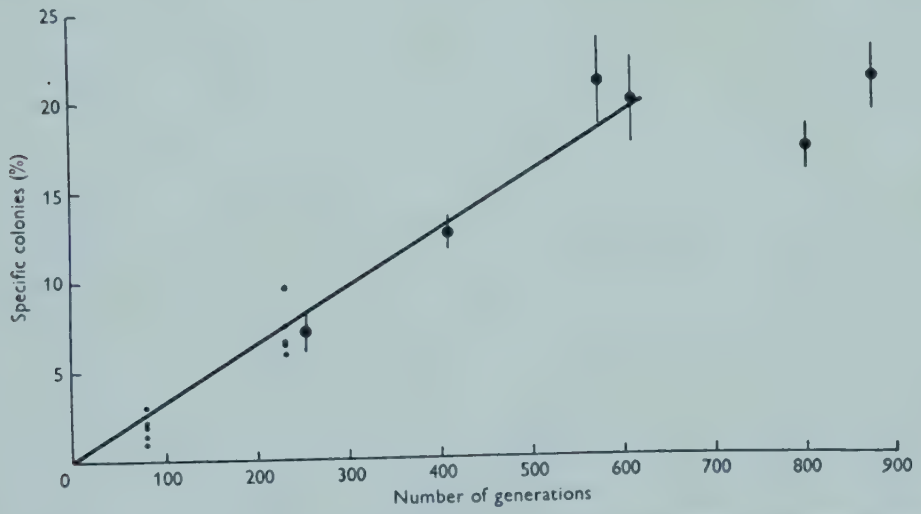


Fig. 46. Increase in the proportion of *specific phase* cells during prolonged logarithmic growth of *S. typhimurium* cultures started with *group phase* cells. Note that the increase is linear up to the 574th generation; thereafter a temporary fall in proportion of specific cells occurs. (From Stocker.[2])

generation of continued growth and a renewed increase in their proportion after an additional 200 generations (Fig. 46). He assumed that this may have been due to the appearance of a faster growing mutant with antigenic characteristics similar to those of

the parent cells. Such mutants with shorter generation time would be able to replace the original cells and their slow-growing antigenic mutants fairly rapidly. However, after continued growth the fast-growing cells might give rise to equally fast-growing antigenic mutants which now could establish themselves under mutation pressure and thus the proportion of mutants would begin to rise again. To illustrate this somewhat complex phenomenon let us assume that from slow-growing cells A equally slow-growing mutants B with altered characteristics (e.g., antigenic properties) arise at a constant rate. B cells will gradually replace A cells, proportional to the mutation rate from A→B. After continued growth A′ mutants may arise from A cells, differing from A in growth rate only. A′ will outgrow both the A and the B cells which established themselves in the initially A population and thus cause a decrease of the proportion of B cells. Eventually, fast-growing B′ mutants will arise from the fast-growing A′ cells and because of the constantly occurring mutations from A′→B′, B type cells will again increase in proportion to A type cells. This can be summarized with the help of the following diagram:

$$\begin{array}{ccc} A & \rightarrow & A' \\ \downarrow & & \downarrow \\ B & & B'. \end{array}$$

Atwood, Schneider and Ryan[*] confirmed Stocker's assumptions in studies with histidine-independent (h^+) and histidine-requiring (h^-) mutants of *E. coli*. They observed that during continuous serial transfers of cultures containing initially various proportions of h^- and h^+ cells an equilibrium level between h^+ and h^- was attained but it exhibited periodic fluctuations. Also, the mean equilibrium level in cultures started with primarily h^- cells was not equal to the mutational equilibrium, i.e., $\frac{\text{mutation rate } h^- \rightarrow h^+}{\text{mutation rate } h^+ \rightarrow h^-}$ (see p. 140), but was in favor of the h^- cells. By isolating h^+ cells at various stages of the continued growth and placing them in competition with h^- cells used originally in initiating the serial transfers, it was confirmed that a phenomenon involving the re-

[*] Atwood, K. C., Schneider, L. K. and Ryan, F. J., Proc. Nat. Acad. Sci., 37: 146, 1951.

placement of the original h^- cells by faster growing h^- cells had taken place. A continuous replacement of mutants in the manner outlined in the preceding paragraph was found to take place. The mutation pattern appeared to be as follows:

$$\begin{array}{cccccc} h_0^- & \rightarrow & h_1^- & \rightarrow & h_2^- & \rightarrow h_3^- \\ \downarrow & & \downarrow & & \downarrow & \downarrow \quad \text{etc.} \\ h_0^+ & & h_1^+ & & h_2^+ & h_3^+ \end{array}$$

The average time elapsing between the periodic replacement of one h^+ type by another appears to be 300 generations. The investigators have proposed the term "periodic selection" for this phenomenon. Periodic selection, by permitting the periodic establishment of selectively advantageous, faster growing, mutants resembling the originally predominant type, tends to maintain a high proportion of cells with characteristics similar to those of the initially predominant cells. It thus acts to stabilize bacterial populations under conditions that are not specifically selective for the type of mutants labelled B or h^+ in our examples. This may explain why under laboratory conditions involving continuous transfers on complex media initially prototrophic bacterial populations remain essentially prototrophic, and initially auxotrophic populations will remain predominantly auxotrophic. Periodic selection thus appears to postpone the establishment of mutational equilibria. It must be reiterated that these phenomena apply only when there is no significant selective advantage for the mutants differing from the predominant parent cells in properties other than growth rate, i.e., if there are no selective advantages for the antigenic or auxotrophic mutants arising in the examples discussed above.

Essentially similar information has been obtained in population studies on phage-resistant and phage-susceptible cells growing in the absence of phage in the chemostat.[4] As mentioned previously, the chemostat is a device that permits the continuous growth of bacterial populations under constant environmental conditions and at a constant rate. Under these conditions it was observed that following prolonged growth repeated population change-overs occur, involving the establishment of mutants differing from the previously predominant cells merely in growth rates. At least two such shifts were observed in an experiment extending over 450 successive generations.

[4] Novick, A. and Szilard, L., Proc. Nat. Acad. Sci., *36*:708, 1950.

Population Changes in the Absence of Constant Environmental Conditions

In general, bacterial populations do not grow for prolonged periods under the ideal constant conditions described in the preceding section. Under most laboratory conditions, and probably in their natural environment as well, bacterial populations fail to propagate at constant rates. Exhaustion of nutrients, the accumulation of toxic metabolic products, changes in the pH of the environment due to the metabolic activity of the bacteria, and defense mechanisms of the host in the case of pathogenic bacteria, are some examples of environmental factors which will interfere with continued growth. Therefore, in most cases the propagation of initially homogeneous populations is inhibited long before changes due to mutation pressure can exert themselves. Under conditions of limited growth of the parent cells the selective growth and survival of mutants especially fitted to survive under the existing environmental conditions becomes a major factor in population changes.

ABSOLUTELY SELECTIVE CONDITIONS

The most extreme type of change in environmental conditions involves absolutely selective agents which completely inhibit continued propagation of the original cell-type. Such conditions are ordinarily not encountered as the result of gradual changes in the environment of bacteria but occur when bacteria are placed in the presence of inhibitory agents; for example, antibiotics, phage or disinfectants. We repeatedly referred to such conditions in Chapters 4 and 5. Population changes can occur in the presence of agents that are completely inhibitory for the majority of originally present cells if a few resistant mutants are present at time of exposure to the inhibitor. Theoretically, all subsequent growth should be due to the growth of the resistant mutants only. However, in practice it is sometimes observed that even following exposure to inhibitory conditions the original cells may multiply for a few generations, side by side with the resistant mutants and their progeny. We have discussed such residual growth previously in connection with the problem of determining mutation rates (p. 65).

Population changes following exposure to absolutely selective conditions sometimes may exhibit a lag period between the time of cessation of growth of the original type and the detectable ap-

pearance of renewed growth due to resistant cells. This becomes especially apparent when growth in liquid media is measured turbidimetrically. This delay is due to the period of time required for a sufficient increase in resistant clones from the few resistant mutant cells present at time of environmental change, and has been discussed in more detail on page 60.

RELATIVELY SELECTIVE CONDITIONS

We shall now consider the types of population change most frequently encountered in experimental studies, where constant environmental conditions are not assured and where the resulting shifts in environment may favor the establishment of mutants without completely suppressing the further multiplication of the parent cells. Such conditions exist during the standard type of cultivation in liquid media and in solid media in which growth invariably ceases after a period of time. This cessation of growth is undoubtedly due to changes in the environment, even though the actual restricting factors are not always known; exhaustion of nutrients seems to play a very minor role. In contrast to absolutely selective conditions, relatively selective conditions fail to suppress the growth of the parent cells entirely but provide an opportunity for the establishment of mutants with equal or greater selective value under the prevailing conditions. The coexistence of both parent and mutant cells during periods of population shifts is characteristic of such changes.

POPULATION CHANGES ON SOLID MEDIA

In the course of the development of a bacterial colony on solid media a single cell may give rise to 10^7 to 10^8 cells. Mutants arising at high rates, therefore, may establish themselves within single colonies, provided their growth rates are equal to or greater than that of the unaltered parent cells. If the mutations involved affect morphological, color or fermentation characteristics, such population changes become easily visible on appropriate media. They may lead to variously sectored colonies if the mutants arise during the period of active multiplication of the parent cells.[5]

Depending on the time of occurrence of the mutation during the development of the colony and depending on the growth rate and stability of the mutant cells, colonies may display different types of

[5] Shinn, L. E., J. Bact., *38*:5, 1939.

sectors. Figure 47 shows some examples of non-lactose-fermenting (lac$^-$) sectors in colonies developing from lac$^+$ cells. The lac$^+$ cells were exposed to ultraviolet irradiation to increase the mutation rate.

As pointed out previously (p. 76), certain sectored colonies, especially those displaying half- or quarter-sectors extending to the center of the colony, may not necessarily owe their formation to a mutation that occurred following the propagation of the initial cell from which the colony developed; such sectors may be due to nuclear segregation occurring after multiplication of an initially multinucleate cell containing one "mutated nucleus."

Frequently a mutant has little selective value when it has to compete with the actively growing parent cells and does not find the proper condition for growth until active multiplication of the non-mutant cells has ceased. Under the latter conditions mutant cells may give rise to secondary colonies growing in the form of papillae on the old colony (Fig. 40).

As a rule, subcultures made from secondary colonies or from mutant sectors of colonies will continue to breed true. One exception to this rule may occur when the growth of the mutant cells is dependent on some metabolic product produced by the parent cells. Transfers from the mutant portion of the colony then may not yield any growth in the absence of the parent cells or may cause the preferential growth of a parent cell accidentally carried over to the new medium during the attempted isolation of the mutant clone.

It is probable that metabolites inhibitory for the parent cells but permitting the growth of mutant cells may play a role in the formation of secondary colonies. It has been reported that the constitution of the medium, e.g., the carbohydrate source used, may affect the frequency of formation of secondary colonies of *Bacillus megatherium*.[6] Such effects are similar to those observed in liquid media where, as we shall see later, changes in the nutrient source may affect the rate of population changes by causing quantitative and qualitative changes in the production of metabolites selectively favoring the establishment of mutant cells. The nature and role of metabolites affecting population changes on solid media have been insufficiently investigated so far.

In general, population changes are less frequent on solid media

[6] DeLamater, E. D., Cold Spring Harbor Symp. Quant. Biol., *16*, 381, 1951.

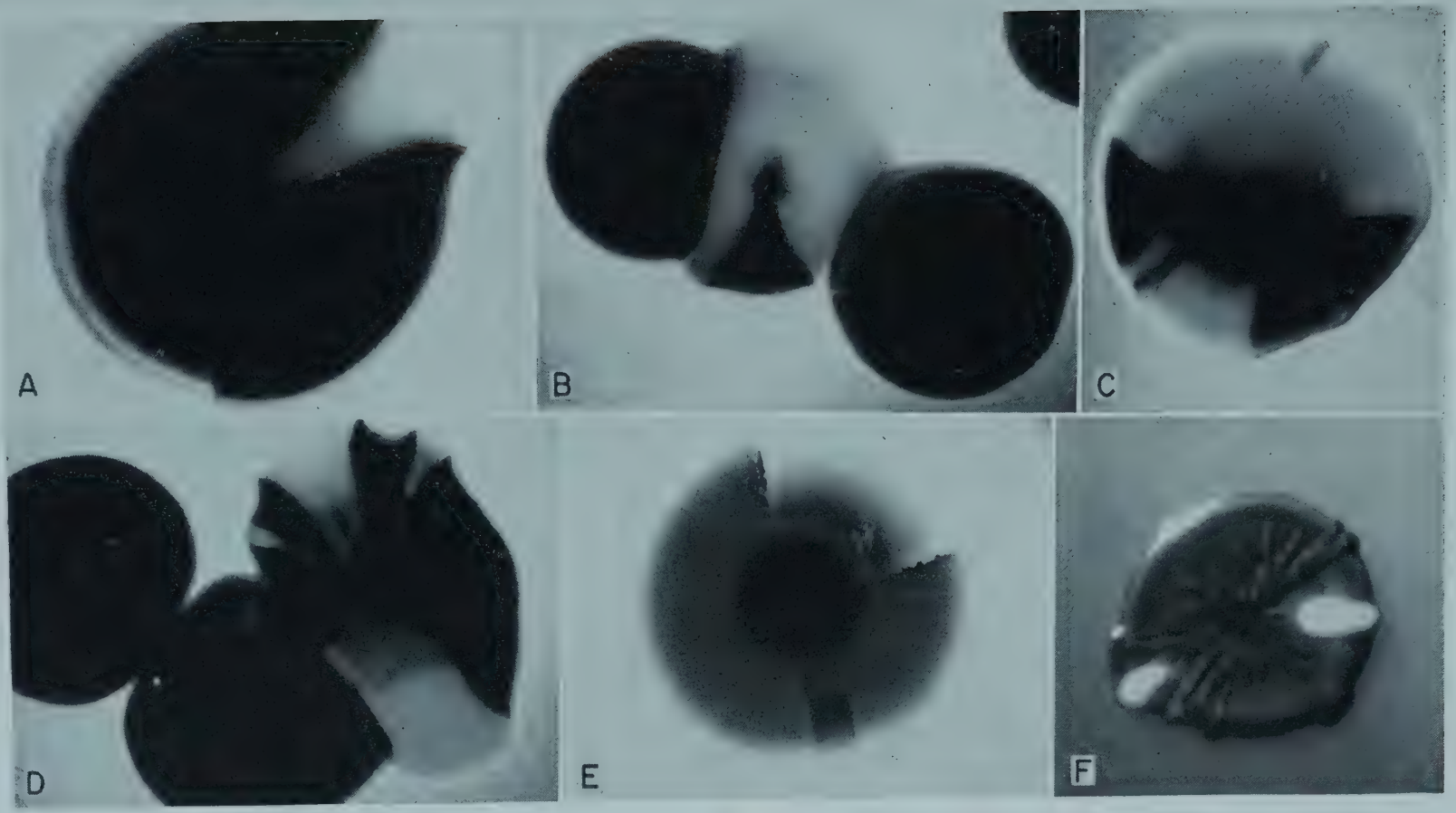


Fig. 47. Sectored colonies of *E. coli* which developed after exposure of lac$^+$ cells to ultraviolet irradiation. Cells were plated on EMB agar. *A-D*, Various lac$^-$ mutant sectors. *E* and *F*, double sectoring: *E* shows a dark and a light mutant sector, neither of which would be scored as lac$^-$; *F* shows sectoring for colonial morphology mutation and for mutation to lac$^-$ (the white edge on the upper side of the colony is due to reflection from the lamp used for illumination.) (H. B. Newcombe.)

compared to changes that may occur in populations of comparable size maintained in liquid media. The factors contributing to this improved stability of populations maintained on solid media are not too well understood but they must include the decreased effectiveness of metabolites that may affect population changes. The latter difference can be illustrated by the fact that in broth cultures of *Brucella abortus* 100 μgm ml of DL-alanine, a normal metabolite, suffices to inhibit the multiplication of smooth cells, whereas more than 10 times this concentration is required to cause a similar inhibition of smooth cells growing on solid media containing 2 per cent agar.

The improved stability of bacterial populations on solid media makes such media the preferred substrate for maintenance of stock-cultures. Also, the preparation of inocula from single colonies is a highly desirable procedure, since in most cases there is a relatively good probability that cells recovered from one colony are more similar genetically than an equal number of cells taken from different colonies or from a liquid culture.

POPULATION CHANGES IN LIQUID MEDIA

Typical growth cycle

The typical laboratory culture of bacteria maintained in a test tube or flask in an appropriate liquid medium at proper temperature displays a characteristic growth cycle. In discussing this cycle we have to distinguish between two types of cell count: (1) the *viable cell count* which measures the number of cells capable of reproduction and which can be obtained by determining the number of colonies which develop after plating of appropriately diluted cell suspensions, and (2) the *total cell count* which can be determined by direct counts of aliquots in a counting-chamber under the microscope or, less accurately, by turbidimetric measurements. Figure 48 illustrates the usual course of the viable and total cell counts after inoculation of a liquid medium which, as is usually the case, does not supply the optimum growth conditions for the inoculated cells. Following a lag period both viable and total cell counts will increase equally and logarithmically. After this period of logarithmic growth the viable cell count reaches a maximum which is retained as long as growth persists. During this period the total cell count may continue to increase at a gradually decelerated rate until the nutrient supply is exhausted.[7]

[7] Jordan, R. C. and Jacobs, S. E., J. Bact., *48*:579, 1944.

Population pressure

At the point of population growth where the total number of cells becomes steadily greater than the number of viable cells, population pressure will start. After this point not every cell that arises will be capable of further multiplication. Therefore, population pressure can act as selection pressure permitting the establishment of any mutant that possesses a faster growth rate or better "viability" under the existent environmental conditions. The re-

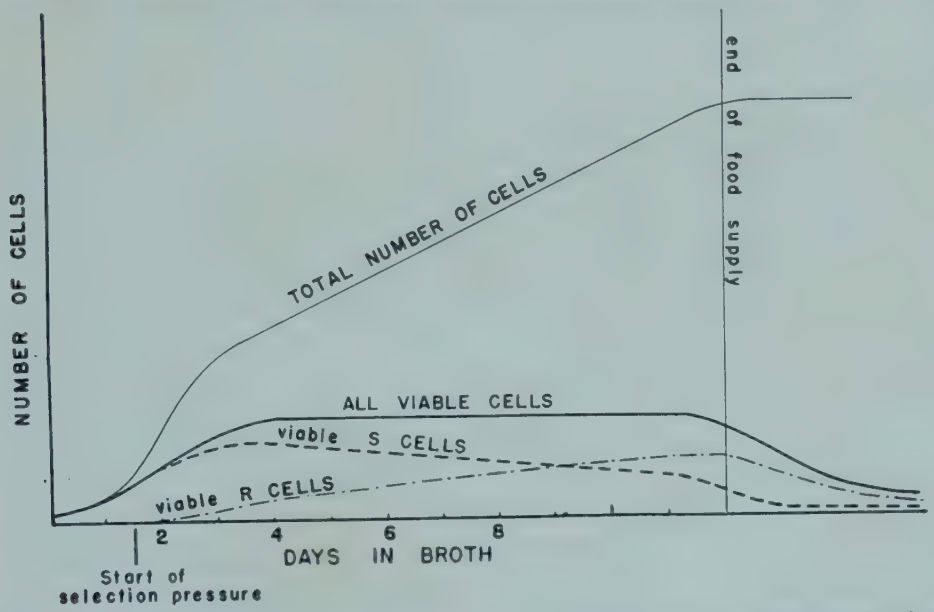


Fig. 48. A graphic representation of the total number of cells and of the number of viable cells during growth of bacterial populations in a closed system and after cessation of growth due to exhaustion of food supply. (From Braun, J. Bact., *51*:327, 1946.)

placement of the original members of the population by mutants with faster growth rates is easily comprehended, but the importance of the differential between the total number of cells and the number of viable cells (viability) may need some explanation. As an example, let us assume that for the original members of a population two out of every six cells arising under given conditions retain the ability to propagate, whereas two out of every three cells of a spontaneously arising mutant propagate under the same environmental conditions. Since the total viable population usually is limited to a maximum number per unit of the environment, the higher "viability" of the mutant will suffice to replace the original type even though the mutant type may actually have a slower growth rate.[8]

[8] Braun, W., J. Bact., *51*:327, 1946.

Factors influencing the rate of population changes

We thus can list several inherent factors that can influence the rate of population changes in a bacterial culture under restricted growth conditions:

1. Rate of mutation from the original type to a type better "adapted" to the existent environmental conditions.
2. Growth rate of the mutant type in relation to that of the parent type.
3. Viability of the mutant type in relation to that of the parent type.
4. Growth rate of the parent type.
5. Viability of the parent type.

Both growth rate and viability of the parent type affect the time at which population pressure starts, and since mutants find little opportunity to establish themselves prior to this period, a shift in the time of onset of population pressure will affect the rate of population changes. The factors listed under 2 to 5 are capable of modifying the rate of population changes even in the presence of constant mutation rates. Any environmental agent capable of modifying these factors can also affect the rate of population change.

Experimental data supporting such considerations have been obtained with various bacteria, but especially in studies on population changes of *Brucella abortus* involving mutants with altered antigenic and colonial characteristics.[9] Following inoculation of smooth cells into broth, population changes involving the gradual establishment of non-smooth mutants usually can be observed after the maximum viable cell count has been reached. The rate and extent of such population changes can be determined by periodic plating of aliquots on solid media and determining the percentage of smooth and non-smooth colonies that develop. Data thus obtained have confirmed that the inherent properties of viability and growth rate, listed under 2 to 5 above, affect the rate of establishment of spontaneously arising mutants. In addition, various environmental conditions which are capable of modifying these inherent properties were found to modify the rate of population changes. In the bacteriological literature on population changes involving mutants with altered antigenic characteristics these facts have frequently been reflected in different terms, such as "a rapidly dissociating culture," or "a strain with slow rate of dissociation," or

[9] Braun, W., Bact. Rev., *11*:75, 1947.

"a high dissociation index (= percentage of non-smooth cells in an originally smooth population after a certain period of incubation)," etc.

The role of metabolites in population changes

As we have seen, the eventual cessation of an increase in the viable cell count occurring in a closed culture system like the test tube can create conditions favorable for the growth and selective establishment of mutants displaying greater viability under the

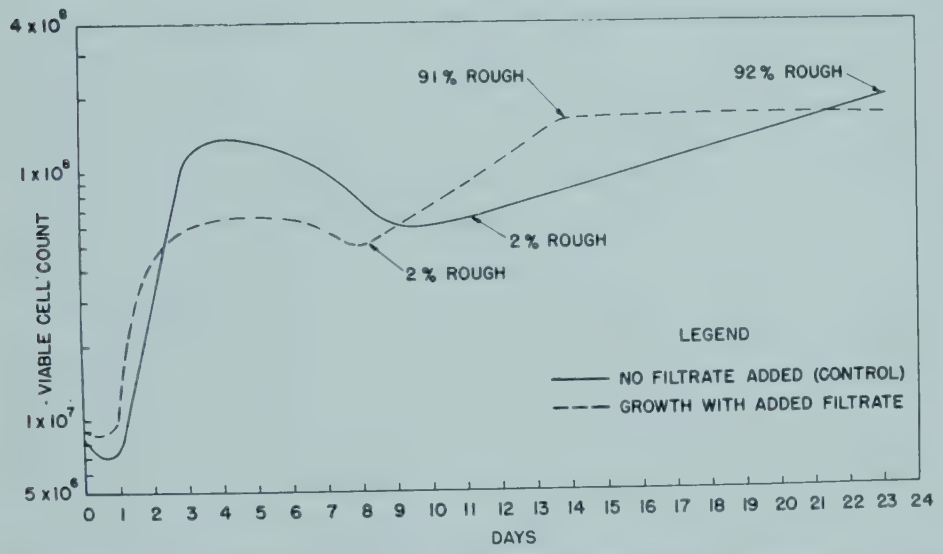

4 x 10⁸
1 x 10⁸
1 x 10⁷
5 x 10⁶
VIABLE CELL COUNT
91% ROUGH
92% ROUGH
2% ROUGH
2% ROUGH
LEGEND
NO FILTRATE ADDED (CONTROL)
GROWTH WITH ADDED FILTRATE
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
DAYS

Fig. 49. Growth (viable cell counts) and population changes in initially smooth *Brucella abortus* cultures maintained in synthetic medium with or without filtrates from old cultures. (From Goodlow, Mika, and Braun.[12])

conditions existing in the culture at the time. Ever since the days of Bail,[10] who labelled the maximum viable count the "M concentration," efforts have been made to identify the actual nature of the factors causing the cessation of logarithmic growth. Several investigators suspected and furnished limited evidence that the accumulation of filterable, toxic factors may be responsible for inhibiting the continued propagation of members of the original population.[11] The most extensive investigation, and perhaps the most clear-cut demonstration, of the role of such inhibitory factors was made possible in studies with *Brucella* species.

[10] Bail, O., Z. Immunitätsf., *60*:1, 1929.
[11] Möller, O., Acta Path. et Microb. Scand., *Suppl. 74*, 1948.

We shall review these data in greater detail now as an example of a general mechanism of population changes that appears to be of widespread occurrence.

Smooth *Brucella* cells growing in a synthetic medium containing DL-asparagine as the sole source of nitrogen displayed the typical logarithmic increase in the number of viable cells which ceased after four days (Fig. 49). During the next five days of incubation the viable count dropped; there then followed a renewed rise in

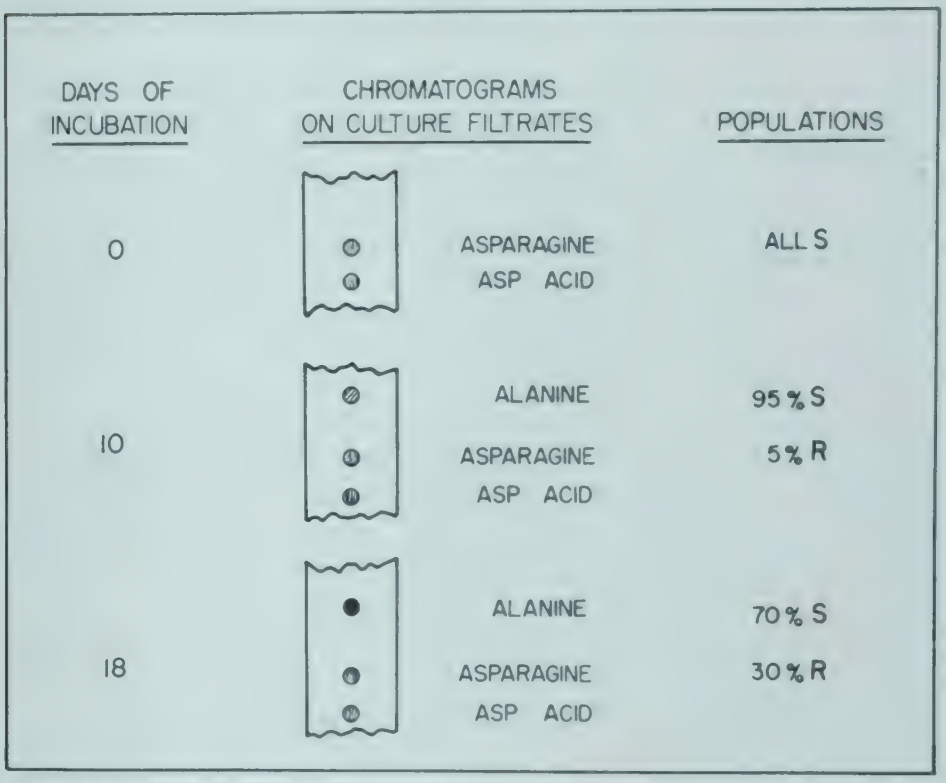


Fig. 50. Alanine accumulation, tested by paper partition chromatography, and population changes in initially smooth *Brucella abortus* cultures.

viable count resulting from the growth of progeny of rough mutants. As Figure 49 illustrates, addition of 10 per cent by volume of a Seitz-filtrate from a 24-day-old culture to a freshly inoculated smooth culture resulted in a marked effect on the subsequent growth and population change. The increase of the viable smooth organisms was repressed, and an earlier and more rapid establishment of rough cells occurred. Analysis of culture filtrates by paper partition chromatography revealed that the amino acid alanine, not previously present in the culture medium, started to accumulate just prior to the time when non-smooth cells started to establish

themselves[12] (Fig. 50). Addition of D- or DL-alanine to smooth cultures caused an inhibition of smooth cells and earlier establishment of non-smooth cells similar to that observed in cultures to which old culture filtrates had been added. Addition of L-alanine had no effect on growth or population changes.[13] It was determined that DL-alanine in a concentration of 100 μgm per ml or more exhibited a significant growth-depressing effect on the smooth cells, whereas the rough cells remained unaffected until a concentration of 1000 μgm of alanine per ml of medium was reached. The significance of these results was underlined by the demonstration that *Brucella abortus* cells produce alanine by direct amination or transamination[14].

The experiments thus revealed that the accumulation of a specific metabolite, alanine, limits the increase in viable smooth cells and at the same time creates an environment favorable for the progressive establishment of non-smooth cells with greater resistance to this inhibitory metabolite. It will be recalled that non-smooth mutants arise in smooth populations at the rate of 1×10^{-7} (p. 124), and we have now seen how such mutants attain an opportunity to establish themselves in originally smooth populations because of their greater resistance to alanine. Prior to population changes in a closed system, such as a test tube, the parent cells can thus create conditions that may be termed "suicidal" and thereby set up specific environmental effects that will favor the establishment of metabolite-resistant mutants. The latter, as illustrated in the case of *Brucella,* may display altered antigenic, colonial and virulence characteristics.

Similar effects of metabolites upon population changes seem to occur in various species. Evidence for this conclusion is available for *Salmonella typhimurium*[15] where the metabolite threonine causes the selective establishment of more virulent mutants with increased threonine-resistance (see p. 169), and *Bacterium tularense,* where an as yet unidentified metabolite affects the establishment of mutants with altered colonial morphology and virulence. Limited evidence indicates that similar phenomena may play a role in population changes of *Mycobacterium tuberculosis, Coryne-*

[12] Goodlow, R. J., Mika, L. A. and Braun, W., J. Bact., *60*:291, 1950.
[13] Goodlow, R. J., Braun, W. and Mika, L. A., Arch. Biochem., *30*:402, 1951.
[14] Altenbern, R. A. and Housewright, R. D., J. Bact., *62*:97, 1951.
[15] Page, L. A., Goodlow, R. J. and Braun, W., J. Bact., *62*:639, 1951.

bacterium diphtheriae, *Shigella dysenteriae*, and crown gall bacteria. In addition, Bunting's data obtained in earlier population studies with color variants of *Serratia marcescens*[16] suggest the possible involvement of a metabolic factor. She observed that in ageing broth cultures mixed populations change in a reproducible manner from cells producing predominantly dark red colonies to pink and white colony-forming cells. It could be shown that in fresh broth conditions prevailed which favored the establishment of cells that developed into dark red colonies, but this initial growth produced changes in the environment which in turn caused an increase in pale variants. An increase of pale variants could be effected in fresh broth cultures by adding autoclaved filtrates of old broth cultures or of autolyzed dark red cells. The actual nature of the responsible factor, however, has remained undetermined.

Apparent cycles and reverse population changes

It was determined in the *Brucella* studies that alanine production continued during prolonged incubation of cultures in which rough cells with greater alanine resistance than that of the original smooth population had become established. When the alanine concentration of the culture liquid reached a level that was inhibitory for these rough cells, still other antigenic mutants with higher alanine resistance established themselves progressively in such cultures. The actual antigenic characteristics displayed by these mutants with increased resistance to a specific metabolite depended upon the strain and species employed. In a *Brucella abortus* strain dwarf rough mutants were found after 60 days of incubation which were not only highly resistant to alanine but actually required alanine for optimal growth. Therefore, in a closed culture system the continuous quantitative and qualitative alteration of metabolite concentration can create a constantly changing environment that successively favors different mutants as long as reproduction continues. The concurrent increase of toxic metabolite level and successive establishment of mutants with higher resistance can cause what appear to be *progressive* population changes, a phenomenon that was regarded as a life-cycle by many earlier bacteriologists.

Again in studies with *Brucella abortus*, a type of population change frequently reported in the older literature was elucidated. It involves population changes from smooth to rough and eventu-

[16] Bunting, M. I., J. Bact., *43*:593, 1942.

ally back to smooth (S⇄R), or more precisely the gradual establishment of R variants in a population originally consisting of S cells only and the subsequent establishment of S mutant cells in this R population. It was determined that the S mutants arising in the R population are similar in colonial morphology, virulence, and antigenicity to the original S cells but differ from them in one important respect: they are more resistant to alanine than either the original S or the R cells.[17] Thus what had appeared to involve reverse changes from S→R→S, actually involved a population change from S→R→S′, where S′ cells simulate the phenotype of the S cells in most respects except the property which gives a high selective value in an aged culture containing a high concentration of alanine. Many older observations recorded as reverse changes, or the establishment of reverse mutants, may represent similar progressive mutational changes. Demerec[18] has reported the same phenomenon in studies involving phenotypic changes from streptomycin-dependence to streptomycin-resistance in *E. coli* (see also p. 96).

The effect of the source of N upon population changes

The recognition of the role of metabolites in population changes has revealed that the parent type can actually influence the direction in which population changes may occur since its metabolic products can determine which of the many spontaneously arising mutants will have the greatest survival value at any given time of a culture's growth. This was confirmed in studies with *Brucella* in which D- or L-asparagine instead of DL-asparagine was used as the source of nitrogen in the liquid synthetic medium.[19] When grown in media containing L-asparagine, most smooth strains yielded a high viable count, failed to produce alanine and failed to show population changes. In contrast, alanine accumulation, an early inhibition of viable S counts, and population changes took place in media containing D-asparagine, similar to what had been observed in DL-asparagine cultures. The most interesting effects were observed when smooth cells of a *Brucella suis* strain were used as inocula for D- or L-asparagine cultures (Table 10). Alanine accu-

[17] Goodlow, R. J., Braun, W. and Mika, L. A., Proc. Soc. Exp. Biol. and Med., *76*:786, 1951.

[18] Demerec, M., Trans. N. Y. Acad. Sci., *12*:186, 1950.

[19] Goodlow, R. J., Tucker, L., Braun, W. and Mika, L. A., J. Bact., *63*:681, 1952.

mulated in the media containing D-asparagine and population changes involving the progressive establishment of alanine-resistant rough cells took place. However, in L-asparagine media no alanine but another amino acid, valine, accumulated and the progeny of valine-resistant mucoid mutants established themselves in the originally smooth population. This represents a clear example of

TABLE 10. *Population changes and metabolism in initially smooth* Brucella suis *cultures maintained in a synthetic medium with* D- *or* L-*asparagine as the sole source of nitrogen.*

CONFIGURATION OF ASPARAGINE	PERCENTAGE OF VARIANTS ON THE FOLLOWING DAYS					AMINO ACID METABOLITES
	5th	8th	12th	16th	20th	
L	0	0	42M*	52M	41M	Valine
D	16R†	55R	84R	90R	97R	Alanine

* M = Mucoid
† R = Rough

how changes in the nitrogen source can qualitatively alter the accumulation of metabolites and population changes.

Additional correlations between metabolite accumulation and population changes

In addition to the qualitative correlation between metabolite accumulation and population changes just described, several examples illustrating quantitative correlations between the accumulation of metabolites and the degree of population changes were observed in the work with *Brucella*.[19] Thus, maintenance of smooth cultures in flasks led to higher yields of viable smooth cells, lesser accumulation of alanine and slower population changes than in comparable cultures maintained for equal periods in test tubes. It is assumed that these differences are due to differences in oxygen tension produced by differences in the surface liquid volume ratio. Similarly, increased aeration, e.g., incubation on a shaker, will reduce the accumulation of alanine and cause a corresponding decrease in population changes. It has also been noted that a reduction of the concentration of DL-asparagine in the culture medium will result in decreased accumulation of alanine and a corresponding reduction in the rate of population changes.

Such data illustrate how apparently minor changes in environmental conditions can have a significant effect upon growth, metabolite production and population changes. They emphasize the necessity for rigid control of cultural conditions in all bacteriological studies, especially in studies that employ cultivation in liquid media beyond the period of logarithmic growth of the original population. Without due attention to such effects it becomes difficult to compare data obtained in different experiments or in different laboratories.

Prior to the recognition of the role of metabolites in population changes of *Brucella* certain environmental agents had been found that suppressed population changes in smooth *Brucella* cultures. Thus the addition to broth cultures of 5 per cent normal serum from *Brucella*-susceptible animals, of 0.03 M pyrophosphate, or of chelating agents such as ethylene diamine tetra-acetic acid prevented the establishment of non-smooth mutants in smooth cultures[20]. However, these agents did not inhibit the growth of non-smooth cells when added to pure R or M cultures. Sera from *Brucella*-infected animals or normal sera from relatively resistant species failed to produce this inhibition of population changes. The suppression of population changes in the presence of certain normal sera or of various chelating agents could be overcome by the addition of surplus Mn^{++} or Mg^{++}. Evidence is now available indicating that these effects also are due to an effect upon metabolite production by smooth cells. It appears that, depending on the strain employed, alanine production is suppressed or additional metabolites with suppressive action upon the alanine effect are produced in the presence of the environmental agents that suppress population changes. The addition of surplus Mn^{++} or Mg^{++} ions restores the normal metabolite accumulation. It has been suggested that trace metals may be required to catalyze the reactions leading to alanine production and that the binding of these trace metals by chelating agents thus interferes with alanine production. Consequently, in the absence of alanine the non-smooth mutants will fail to find the conditions favoring their establishment in initially smooth cultures. The components of normal serum that produce effects identical to those of chelating agents have been identified as subfractions of β- and γ-globulin.[21] Similar effects of normal serum have been

[20] Braun, W., J. Bact., *58*:299, 1949; Cole, L. J. and Braun, W., J. Bact., *60*:283, 1950.

[21] Braun, W., J. Bact., *58*:291, 1949; Cole, L. J. and Braun, W., J. Immun., *64*:111, 1950.

observed with *Staphylococcus aureus*,[22] *Salmonella* species, and *Bacterium tularense*.

As is well known for a great variety of species, enhanced population changes occur in the presence of antiserum against the type initially used as inoculum.[23] It has been observed that in *Brucella* cultures these enhanced population changes again are associated with modified metabolism, in this case enhanced accumulation of alanine. It is noteworthy that antisera can produce an effect upon metabolism and population changes which appears to be entirely unrelated to their content of agglutinating antibodies.

Other previously observed environmental effects upon population changes in *Brucella* such as those produced by changes in temperature, O R potential, pH, or by variation in the composition of different batches of complex broth,[5] may be produced via modifications of metabolite production as well. Similar effects upon population changes have been described for these environmental agents in studies with various bacterial species[23] but the role of metabolites in population changes of these species remains to be established.

Other environmental agents affecting population changes

Various other environmental agents have been reported to affect population changes of diverse bacterial species, frequently favoring the establishment of specific mutants with altered antigenic or colonial characteristics. Many of these agents are not known to be mutagenic and their effects must be assumed to be due to the creation of specific selective conditions favoring the establishment of certain mutants. Again it remains to be established which of these effects are direct, i.e., inhibit growth or viability of the parent type while favoring those of the mutant cells, and which are indirect, acting through metabolites as shown in the *Brucella* studies. The following are examples of such selective agents: phages, tissue extracts, pleuritic and ascitic fluids, certain laboratory media, atabrine, ferric chloride, substances produced by one species affecting population changes of other species, barium chloride, phenol, penicillin, sulfonamides, and 2-methyl-1,4-naphthoquinone.[23] The five compounds last mentioned can cause the selective establishment of small colony mutants in various bacterial species. These mutants

[22] Hoerlein, B. F., J. Bact., *56*:139, 1948.

[23] For references see Braun, W., Bact. Rev., *11*:75, 1947.

are characterized by inherently lowered metabolism, a property which appears to permit their survival under conditions that are generally unfavorable for growth.

Correlations between population changes and inherent differences in metabolism

Under identical environmental conditions different strains, or clones, isolated from apparently homogeneous cultures can display persistent differences in rate of population changes.[8] For example, in the case of *Brucella abortus,* cultivated in beef-extract broth, smooth clones were isolated that consistently yielded 80 per cent non-smooth variants after 10 days of incubation, whereas other clones isolated from the same smooth stock-culture never showed more than 5 per cent non-smooth cells following 10 days of incubation. Such inherent differences in rate of population change were found to be correlated with inherent differences in rate of alanine production. The most striking case of correlation between inherent differences in metabolism and population changes was seen in studies with a certain smooth strain of *Brucella melitensis.*[24] This strain was found to utilize D-alanine. Therefore, when grown under conditions that led to extracellular alanine accumulation and population changes in all other *Brucella* strains tested, it failed to show any accumulation of alanine in culture filtrates, did not display any population changes and continued to increase in viable count long after other strains had reached their maximum viable level. In the presence of non-inhibitory concentrations of penicillin, the metabolism of this *B. melitensis* strain was modified, alanine accumulated in the culture fluid, and population changes occurred involving the progressive establishment of non-smooth mutants with increased resistance to alanine.

Interactions between mutants in mixed populations

The bacteriological literature contains several references to the fact that regardless of the initial proportion of two types, such as S and R cells, in a mixed inoculum, all broth cultures will display the same ratio of types after a relatively short period of incubation.[23] Similar observations were made with *Brucella abortus* and are illustrated in Figure 51. It can be seen that regardless of the initial

[24] Braun, W., Kraft, M., Mead, D. D. and Goodlow, R. J., J. Bact., *64*:41, 1952.

proportion of R cells in cultures inoculated with a mixture of S and R cells all cultures displayed the same proportion of R cells after 10 days of incubation. The cause for the attainment of such a population equilibrium is quite different from that described on page 140, where forward and reverse mutations bring about an equilibrium between mutants and parent type after continuous and prolonged growth involving as many as 500 generations. The type

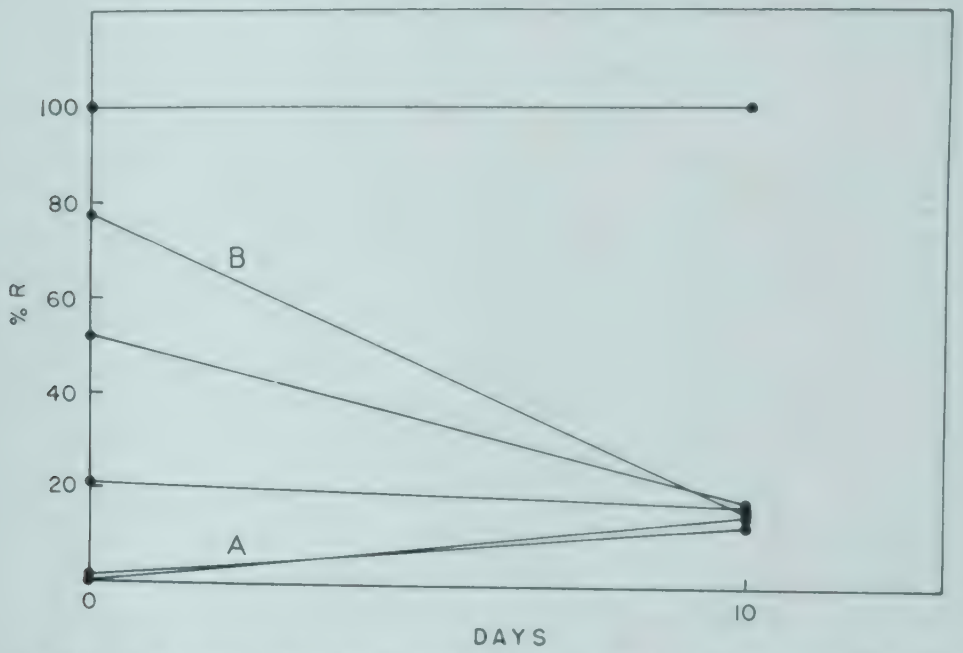


Fig. 51. The percentage of R types in 10-day-old broth cultures of *Brucella abortus* originally inoculated with various mixtures of S and R type cells. Note that the lines connecting the 0-hour and 10-day values are diagrammatic only and do not necessarily represent the true values of the proportion of R types at intermediate points. (From Braun *et al.*[25])

of equilibrium (more precisely, a transient equilibrium) to which we now refer occurs in cultures maintained under conditions of limited growth and is achieved so rapidly that mutation pressure cannot be held responsible. Equilibria such as those illustrated in Figure 51 are apparently due to interactions between the two cell types involving their respective rates of production of an inhibitory metabolite and differential resistance to this inhibitory substance. This has been shown to be the case with *B. abortus*[25] where S and R types differ not only in their susceptibility to the inhibitory effects

[25] Braun, W., Goodlow, R. J., Kraft, M., Altenbern, R. and Mead, D., J. Bact., *62*:45, 1951.

of alanine, but also in their rate of alanine production. S cells produce alanine at a greater rate than R cells and are more sensitive to it. Thus, a culture which initially contains a high percentage of S type cells (line A in Fig. 51) will soon attain an alanine level inhibiting S cells and favoring the establishment of R cells. Consequently the percentage of R types will show a significant increase during a 10-day period of incubation. In contrast, there is much slower alanine accumulation in cultures which initially contain a high percentage of R cells. In the absence of high alanine levels it is the faster growing S type which is selectively favored over the R type; consequently the percentage of R type cells will decrease in such cultures during the initial 10-day period of growth (line B in Fig. 51). These population changes can take place very rapidly as indicated in Figure 52. Viable cell counts of homogeneous S and homogeneous R cultures grown in the absence or presence of DL-alanine have confirmed that S cells have a greater survival value than R cells in the absence of significant alanine levels, whereas R cells yield higher viable counts than S cells in the presence of alanine. Such data serve to demonstrate that the selective value of any given mutant may differ under different environmental conditions. It is noteworthy that in the well analyzed case of population changes in *Brucella* this shift in selective values can be referred to environmental changes produced by the cells themselves.

It has also been established in these studies that the level of the early population equilibrium can be affected by a number of factors: the genotype of the cells, the environmental conditions existing in the mixed culture, and the conditions under which the inoculum has been grown previously. Thus, when different S clones are used the level of the equilibrium differs, an effect that can be attributed to clonal differences in the rate of alanine production. A similar shift in the S/R equilibrium can also be produced by environmental influences. For example, initial addition of DL-alanine to mixed S + R cultures results in S/R equilibria with a larger proportion of R cells than those found in control cultures without added alanine (Fig. 52). When agents that interfere with alanine accumulation, such as pyrophosphate, are added to the cultures, the S/R equilibrium will be shifted in favor of S type cells.

Shifts in the equilibrium also occur when the S cells used are pregrown in different media.[25] Thus mixed S + R cultures in standard broth, prepared from S cells pregrown for three days in a

medium enhancing alanine production, will show a higher proportion of R cells in the S R equilibrium than corresponding cultures prepared from S cells pregrown in a standard medium. Similarly, the S R equilibrium is shifted in favor of the S cells if, prior to growth in the standard medium, the latter are pregrown in a medium inhibiting alanine production. Such "carry-over" effects are prob-

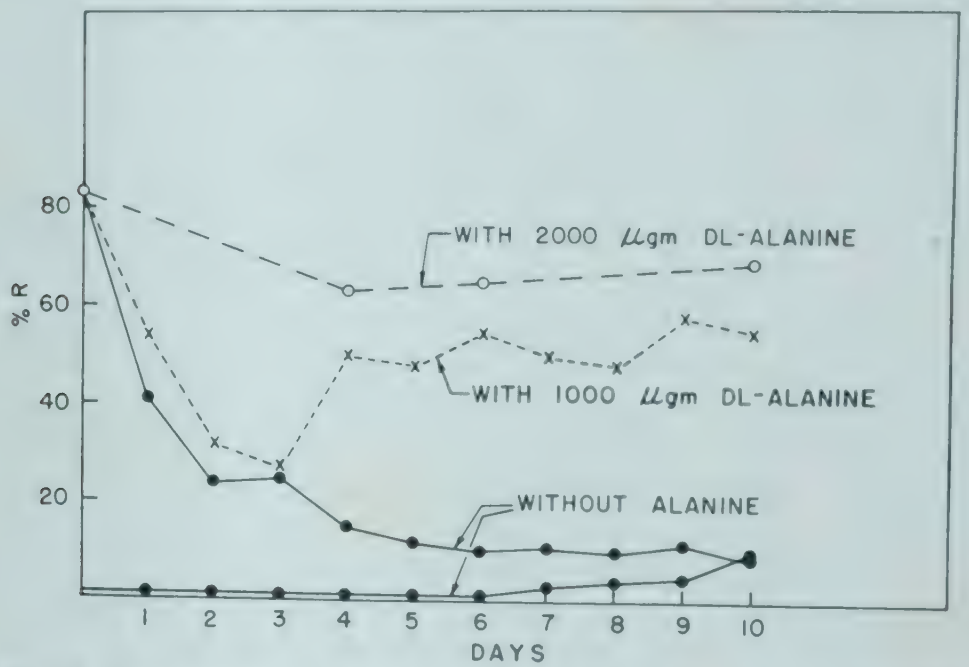


Fig. 52. Daily changes in proportions of S and R types in broth cultures of *Brucella abortus* started with mixed inocula, and the effect of initial addition of DL-alanine upon such population changes. (From Braun *et al.*[25])

ably due to the ability of cells to store metabolic products and to utilize or to excrete them for a number of generations after transfer to a new environment. Wherever metabolic products play a role in population changes such effects must be kept in mind; environmentally induced temporary modifications of metabolism may persist for limited periods after transfer to new environmental conditions, and the degree of early population changes can thus be modified, depending on the previous growth-conditions.[26]

[26] Recent studies (Mika, L. A., Braun, W. and Goodlow, R. J., Bact. Proc., 1951, p. 79) have supplied some information on the nature of the inhibitory effects of DL-α-alanine which have been described in the preceding sections. It appears that *Brucella* cells require pantothenic acid for efficient growth and are able to synthesize this compound from pantoic acid and β-alanine. When α-alanine accumulates in the culture, the cells appear "to make a mistake," that is, they start to couple α-alanine instead of β-alanine

Population changes involving inhibitory effects produced by the parent type upon mutant cells

In contrast to phenomena described above where the establishment of mutants is *favored* by metabolic products of the parent type, the opposite type of effect can occur, i.e., the propagation of mutants may be *inhibited* by the growth of the parent cells and their metabolic products. Ryan and co-workers[27] analyzed such a case in a series of studies with a histidine-requiring (h^-) strain of *E. coli* and a histidine-independent (h^+) mutant derived from it. It was shown that the establishment of h^+ mutants in an h^- culture (also referred to as "adaptive growth") is dependent on the activity of h^- cells. As one might expect, in the presence of suboptimal concentrations of histidine, selection is strongly in favor of h^+ cells since h^- cells cease to grow when the histidine content of the culture is exhausted. At that time the h^+ component of the culture continues to grow, but the extent to which such growth proceeds depends upon the activity of the h^- bacteria in the stationary phase. In aerated cultures the h^- cells continue to consume glucose in the stationary phase and the exhaustion of glucose causes a cessation of growth of h^+ cells. In unshaken cultures the glucose supply does not become limiting, but the h^- cells in the stationary phase continue to produce acid and an inhibitor of unknown constitution, active at any pH range, both of which limit the amount of growth of h^+ cells. Thus the establishment of h^+ cells in h^- populations can be inhibited by different means (the production of acid and an inhibitor or the exhaustion of glucose) all of which involve a modification of the medium by bacteria in the stationary phase. These findings explain why h^+ cells establish themselves rapidly in h^- populations with initially low histidine concentration, but fail to establish themselves at high histidine concentrations. The amount of histidine determines the size of the stationary population of h^- bacteria, and the more h^- bacteria in the stationary phase, the sooner will the medium fail to support growth of h^+ organisms.

Similar inhibitions of the growth of mutant cells in the presence of inhibitory conditions produced by cells of the original popula-

with pantoic acid and thus produce a compound that they cannot utilize. This inhibition of pantothenic acid synthesis by α-alanine appears to be similar to the interference produced by sulfonamides in the synthesis of para-aminobenzoic acid.

[27] Ryan, F. J. and Schneider, L. K., J. Bact., *56*:699, 1948; *58*:181, 1949; *58*:191, 1949; *58*:201, 1949.

tion have been reported but have been less extensively analyzed by other investigators. For example, Zamenhof,[28] investigating the establishment of citrate-fermenting mutants of *E. coli* in populations of non-fermenters, has claimed that the mutants are specifically inhibited by some unidentified product of the metabolism of the parent type. Guthrie[29] has reported that the frequency of reversion of cultures of a purine-requiring *E. coli* mutant is greater in low concentrations of purine when the number of parent bacteria is lower. Also, inhibition of phage-resistant or streptomycin-resistant mutants of *E. coli* in the presence of increasing concentrations of sensitive parent cells has been observed.[30]

Determination of the selective value of a mutant

Examples like the foregoing, as well as the type of interaction described on page 160, clearly illustrate that it is not always possible to determine the selective value of a mutant by comparing its growth and viability in pure cultures with those of the parent type grown in pure culture. So many examples are now known of significant modification of selective values when a mutant grows in association with its parent type that inferences from data on separate growth to behavior in mixed populations become at least questionable. Nor can evidence derived from artificial mixtures of mutant and parent types be entirely relied upon. The recent literature on mutagenic agents contains several examples where investigators, in order to demonstrate the absence of selective values of certain mutants, followed the fate of the mutant type cells in artificial mixtures of parent and mutant cells. It is evident that conclusions from such "reconstruction experiments" may not necessarily be valid since, as illustrated in Figure 51, different initial proportions between two types may result in different, even opposite, selective value. In addition, since growth of one cell type can continuously modify the environmental conditions, the relative selective value of a mutant may differ depending on the time in a culture's growth at which it is introduced or arises. Thus, it would be extremely difficult to reconstruct accurately selective conditions that exist under natural circumstances when a mutant may occur at different stages of the culture's growth as approximately 1 cell among 10^8 parent cells.

[28] Zamenhof, S., J. Bact., *51*:351, 1946.

[29] Guthrie, R., J. Bact., *57*:39, 1949.

[30] For example, see Bertani, G., Genetics, *36*:598, 1951.

CHAPTER 9

Population Changes Due to Mutation and Selection: II

THE EXPERIMENTAL DATA DESCRIBED IN THE PRECEDING CHAPTER clearly indicate that bacterial population changes can be caused by mutation and selection. This conclusion is further supported by the available information about the behavior of bacterial populations in their natural habitat. Such information will be reviewed now. In addition, some of the relationships between population dynamics and problems of bacteriological procedures will be discussed in the present chapter.

POPULATION CHANGES *IN VIVO*

A considerable number of reports attest to the fact that heterotrophic bacteria may undergo population changes within the host environment. It is common experience that such population changes *in vivo* may differ from those observed *in vitro* as to both rate and mutant type selected. Factors that can account for some of these differences include: (1) the presence of specifically selective host factors, such as tissue factors, and, in the case of pathogens, defensive factors, including antibodies; (2) the usual lack of a closed system in which inhibitory metabolic products of the bacteria, affecting the survival value of parent cells or mutants, could accumulate; (3) the availability of a constant supply of nutrients which may permit a pattern of growth resembling that occurring in a chemostat (p. 139) rather than that observed in test tubes.

Examples of population changes *in vivo*

Except for the reports on the establishment of drug-resistant bacterial populations in patients under treatment (see p. 87), most investigations on population changes *in vivo* have concerned themselves with changes involving mutants with altered virulence and antigenic characteristics. The following are typical examples.

It has been reported that after inoculation of I type cells of avian or human tubercle bacilli into hens, populations containing S cells subsequently could be isolated from the host, whereas predominantly R populations were isolated following passage of these strains through guinea pigs.[1] In other studies with avian tubercle bacilli it was noted that isolations from chickens always yielded S populations regardless of the antigenic type inoculated, whereas isolations from rabbits generally were of the same antigenic type as that inoculated.[2] Population changes involving the establishment of virulent S mutants have been recorded following the inoculation

TABLE 11. *Comparison between selective effects in chicks and guinea pigs inoculated with S, M, and R mutants of* Brucella suis. *Number of cells inoculated intraperitoneally into each animal: 2 x 10^9.*

HOST	INOCULUM: 51% M + 49% S % M recovered from spleens of individual animals after 1 week			INOCULUM: 48% R + 52% S % R recovered from spleens of individual animals after 1 week		
Chick	99	93	32	66	98	92
Guinea pig	<0.01	<0.01	<0.01	<0.1	<1	<0.01

of relatively avirulent M cells of *Brucella suis* into guinea pigs.[3] These and similar observations[4] indicate that in the case of pathogenic bacteria the virulent type is most apt to establish itself within susceptible hosts, a tendency which is easily understood since virulence is usually associated with properties that appear to protect these types from the defense mechanisms of the host and thus endow them with high selective values. For example, it has been demonstrated repeatedly for various species that relatively avirulent, non-smooth cells are far more easily phagocytized than virulent S cells. In contrast to this selection against mutants with decreased virulence in susceptible hosts, mutants with lower virulence seem able to maintain themselves in resistant hosts. For example, after inoculation of mixed suspensions of S + M cells of *Brucella suis* into highly susceptible embryonated eggs, population changes involving the disappearance of the less virulent M cells occur rapidly.

[1] Buonomini, G., Bull. assoc. diplômes microbiol. faculté pharm. Nancy, *16*, 17, 1938.

[2] Ferreira, A. J., Arquiv. Inst. bact. Camara Pestana, *8*:133, 1942.

[3] Jones, L. M. and Berman, D. T., J. Inf. Dis., *89*:214, 1951.

[4] For further references see Braun, W., Bact. Rev., *11*:75, 1947.

In contrast, mixed S + M populations are maintained for prolonged periods after inoculation into more resistant chicks[5] (Table 11). This ability of mutants with decreased virulence to persist in normally resistant hosts is paralleled by various observations on the establishment of relatively avirulent mutants in immunized hosts and in asymptomatic carriers.[4]

The nature of selective host factors

The actual nature of the host factors that may exert a specific selective effect upon bacterial population changes *in vivo* has been determined in a few cases only. In studies with *Brucella* it has been possible to recognize a serum factor which is present in susceptible animals and prevents population changes without affecting growth when added to virulent smooth cultures *in vitro*. Sera obtained from resistant species and from immunized or infected animals of susceptible species do not produce this selective effect but permit the rapid establishment of relatively avirulent non-smooth mutants during cultivation *in vitro* (see p. 157). At one time it was believed that these serum effects might play a significant role *in vivo* as well as *in vitro*. However, repeated trials failed to confirm this.[3,6] Non-smooth mutants could not be isolated with any consistency from guinea pigs infected with smooth cells even during periods when these animals lacked the selective serum activity. This result is understandable in view of the demonstration *in vitro* that the accumulation of alanine is responsible for the selective establishment of non-smooth mutants in originally smooth cultures, and the finding that the selective serum factor prevents population changes by interfering with the production of alanine by the cells. A similar accumulation of alanine presumably does not occur *in vivo* since the host presents an "open system" from which bacterial metabolites can be removed as efficiently as metabolic products of the host. Therefore, in contrast to the situation during cultivation *in vitro*, conditions favoring the establishment of metabolite-resistant mutants with altered virulence normally are not created within the animal. However, it is interesting to note that non-smooth *Brucella* mutants have been recovered from walled-off abscesses of animals originally infected with smooth cells.[6] These abscesses contained a

[5] Kraft, M. and Braun, W., Bact. Proc., 1952, p. 78.

[6] Braun, W., Gorelick, A., Kraft, M. and Mead, D., J. Inf. Dis., *89*:286, 1951

significantly higher level of amino acids, including alanine, than the surrounding "open" tissue. Thus it appears that population changes similar to those occurring in test tubes may occur *in vivo* when bacteria find themselves within a closed environment such as is represented by walled-off abscesses.

It is evident that the factors contributing to population changes *in vivo* are highly complex. Not only does the establishment of mutants with altered virulence *in vivo* depend on the susceptibility of the host and its ability to support or to inhibit the growth of the parasite in general and that of specific mutants in particular, but it appears also that the specific site of propagation may influence population changes.

The effect of amino acids upon population changes *in vivo*

It has been established in a few cases that specific amino acids can affect population changes not only *in vitro* but also *in vivo*. Thus alanine, which in the case of *Brucella* is associated with the selective establishment of more alanine-resistant mutants with altered virulence *in vitro*, can cause *in vivo* population changes of *Brucella*.[7] It was found that the administration of alanine (400 mg per day for 14 days) to guinea pigs infected with S cells resulted in an increase of virulence due to population changes involving the establishment of more virulent, more alanine-resistant S′ mutants. Alanine-resistant R mutants, which had been found consistently after accumulation or addition of alanine in S cultures maintained *in vitro*, were not observed in isolates from alanine-treated animals. Such R mutants, although they may arise *in vivo*, presumably have little chance to establish themselves in animals because of their greater susceptibility to phagocytosis.

A similar modification of virulence due to population changes *in vivo* under the influence of a specific amino acid has been reported for *Salmonella typhimurium*.[8] It was observed that, after infection of guinea pigs with 4×10^5 cells from a stock culture of *S. typhimurium*, 60 per cent of all animals died within 15 days, whereas 100 per cent died when animals were injected with 50 mg of DL-threonine at time of infection. Also, the average time of survival after infection was significantly shorter when threonine was given, compared to the time elapsed before death occurred in ani-

[7] Mika, L. A., Goodlow, R. J. and Braun, W., Bact. Proc., 1952, p. 77.
[8] Page, L. A., Goodlow, R. J. and Braun, W., J. Bact., 62:639, 1951.

mals that did not receive threonine. This enhanced virulence after injection of threonine was found to be due to the *in vivo* selection of a more virulent, threonine-resistant mutant that had been present in the original stock culture. It was established that the *Salmonella* suspensions used for inoculation into the animals contained less than 1 per cent of such threonine-resistant (S^t) cells, in addition to threonine-susceptible (S^o) cells (Fig. 53). S^t and S^o types also

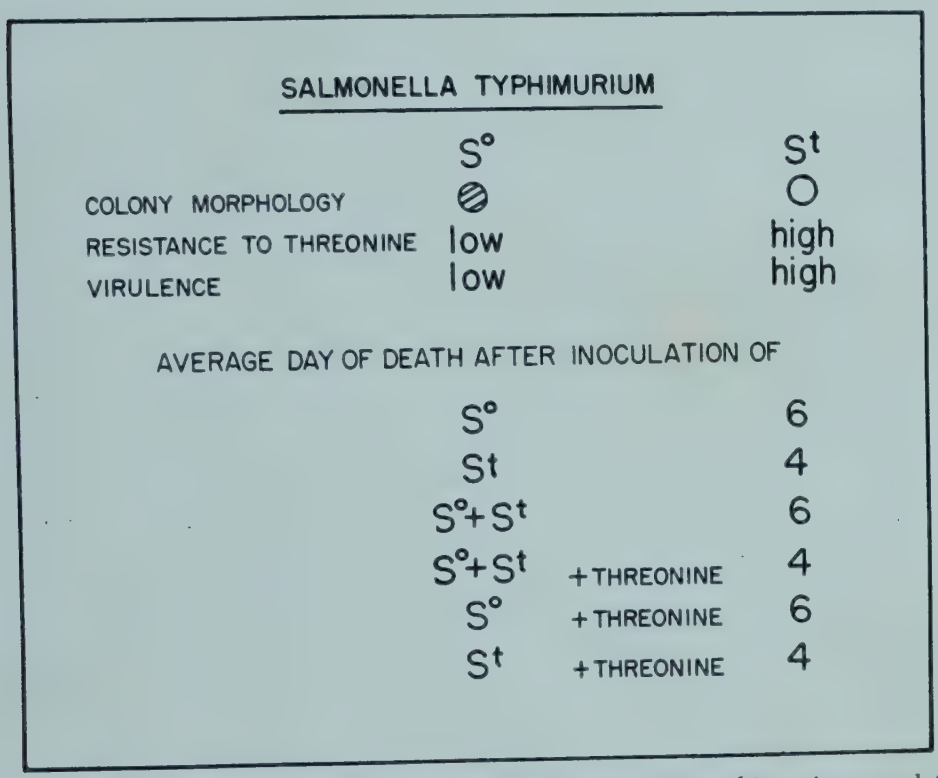


Fig. 53. Properties of S^t and S^o types of *Salmonella typhimurium*, and the effects of threonine upon the severity of infection in guinea pigs infected with approximately 1 x 10^6 cells of these types.

differ in colony morphology (Fig. 39) but are identical in their antigenic and in almost all of their biochemical reactions. *In vitro*, S^t mutants, arising at the rate of approximately 1 x 10^{-6}, established themselves rapidly in originally S^o populations. Similarly, the proportion of more virulent S^t cells increased rapidly in threonine-treated animals infected with mixtures of S^t and S^o, whereas S^o cells predominated at the time of death in similarly infected animals that received no threonine. The increase in virulence of *S. typhimurium* in threonine-treated animals, therefore, can be accounted for on the basis of originally heterogeneous populations containing small num-

bers of more virulent, threonine-resistant S' type cells which will be selectively favored in threonine-treated animals. It is interesting to note (see lower part of Fig. 53) that threonine injections failed to produce an effect on survival when animals were infected with homogeneous S" populations. This result, which is in contrast to the experience with S" populations containing a few S' cells, has been explained on the basis that average time of death is too rapid (6 days), even after infection with the less virulent S" cells, to permit the selective establishment of S' mutants which arise at a relatively low rate. However, if a few S' cells are present initially in S" populations, they will be selected immediately after infection of threonine-treated animals. Such differences in results after use of initially homogeneous or heterogeneous populations of bacteria may account for many controversial observations[9] regarding the effects of diet on infection. Schneider[10] previously demonstrated that nutritional modifications of *Salmonella* infections can be achieved only if inocula (and hosts) are genetically heterogeneous.

MUTATION AND SELECTION IN RELATION TO SOME PROBLEMS OF BACTERIOLOGICAL TECHNIQUE

The recognition that a bacterial culture is not just a conglomeration of similar cells that metabolize and propagate, but represents a population that is constantly subject to changes in inherent properties, demands certain precautions in the execution of bacteriological studies. We already have referred to some of these applied problems during the description of specific phenomena (pp. 148, 157); we shall reiterate some of them here and add a few others.

Maintenance of bacterial stock cultures

Each period of growth provides an opportunity for the occurrence and establishment of mutants. Consequently, in preparing stock cultures a growth period as brief as feasible prior to storage under conditions unfavorable to growth should be employed. In general, the time of pre-storage incubation should not exceed the period of logarithmic growth. In addition, media least favorable for population changes should be used. In this respect solid media are superior to liquid media. Frequent transfers should be avoided, since each growth period furnishes renewed opportunity for muta-

[9] See Clark, P. F., Ann. Rev. Microbiol., *4*:343, 1950, for references.
[10] Schneider, H. A., Am. J. Pub. Health, *39*:57, 1949.

tion and selection of new or already present mutants. If a series of seed cultures is required it is far more advantageous to prepare and store a large series of simultaneous subcultures from one parent culture instead of making repeated serial transfers. Storage at low temperature is always preferable to storage at elevated temperatures, e.g., room temperature, even if the bacteria are suspended in a medium that does not support growth. If the latter procedure is employed, as has been the case in the storage of certain live vaccines, renewed growth may eventually ensue because of the supply of growth factors from dying cells. This renewed growth may furnish mutants with a selective opportunity for growth and can lead to rapid population changes. Theoretically, storage in the frozen-dried state should be the ideal condition for the maintenance of bacterial cultures. However, it has been observed with bacteria[11] as well as with fungi[12] that after reconstitution of apparently homogeneous lyophilized cultures a small percentage of previously undetected variants may be present. The reason for such changes following lyophilization is still undetermined; it may involve a mutagenic effect of freeze-drying or may be due to the destruction of an inhibtor which had prevented the growth of mutants already present in the population prior to lyophilization. (Experimental studies have indicated that the effect is not due to selective killing.) This effect of lyophilization should not prevent its use as a desirable technique for the preservation of bacterial cultures. But it suggests that single colony isolates, insuring the reisolation of the predominant parent type, rather than mass-cultures should be employed after reconstitution of lyophilized cultures for the preparation of new inocula.

In general, transfers made from single colony isolates increase the probability of maintaining homogeneous cultures, since even in the presence of a small proportion of mutant type cells in the parent culture it is more likely that these cells are excluded in transfers made from single colonies. Mass transfers are apt to carry over such mutant cells and thus preserve existing heterogeneity as well as enhance the potential degree of cultural heterogeneity by giving these mutant cells further opportunity to establish themselves during subsequent growth periods. However, since *unselected single colony transfers* may occasionally result in the accidental isolation of a

[11] Braun, W., in *Brucellosis*, Publ. by AAAS, Washington, D.C., 1950.
[12] Subramaniam, M. K. and Prahlada Rao, L. S., Experientia, 7:98, 1951.

mutant colony, it is advisable to check, whenever possible, the properties of cells belonging to the colony which is used for the transfer.

Media providing the desired type with optimal growth conditions should be employed in order to reduce the opportunities for improved survival and growth of undesirable mutants.

Preparation of inocula

The majority of stock cultures propagated over a period of time by arbitrary mass transfers can be assumed to be fairly heterogeneous. Since this genetic heterogeneity may involve all types of characteristics (e.g., growth rate, nutritional requirements, metabolite production, fermentative, antigenic and virulence characteristics), it is often difficult to ascertain the degree of heterogeneity. However, as pointed out repeatedly in preceding chapters, colonial morphology on transparent solid media, viewed with oblique lighting, constitutes a simple indicator for many organisms of the relative degree of heterogeneity, since many types of mutational changes appear to produce changes in colonial morphology. For the preparation of inocula from any stock culture it becomes again desirable to utilize material grown from single colony isolates (keeping in mind, however, the dangers of unselected single colony transfers pointed out above). Although this does not necessarily result in the isolation of a clone, especially when practiced with species in which cells tend to associate in clumps or chains, it is still likely to reduce any existent heterogeneity. Such single colony isolates should be made from colonies that have not reached their maximal size. Furthermore, under conditions of growth that lead to a maximal level of viable cells, i.e., maintenance in test tubes or flasks without transfer, the size of the inoculum must be taken into consideration. The number of cells in the inoculum can affect potential population changes, because the larger the inoculum the earlier will competitive conditions be attained in the culture.

Unless efforts are made to control the homogeneity of inocula for cultures or animals erroneous conclusions can easily result. This is exemplified in results obtained in studies on the immunogenic potency of *B. tularense* cultures[13] where it was demonstrated that immunogenic differences between cultures could be attributed to the degree of heterogeneity existing within the cultures under comparison. In other words, differences which had been observed in

[13] Eigelsbach, H. T., Braun, W. and Herring, R. D., J. Bact., *61*:557, 1951.

immunogenicity of various cultures were found to be due to the relative proportion of highly immunogenic and weakly immunogenic cells in the heterogeneous cultures. The identification of mutants with different immunogenic potency was made possible by the detection of colonial differences among these mutants (see Fig. 38). In work with various other species, a similar heterogeneity has been detected in cultures used for the preparation of vaccines. In every case, subsequent isolation and maintenance of the highly immunogenic component of the population resulted in greatly improved vaccines.[4]

Similar considerations apply to *in vitro* work with bacteria, for example, metabolic studies. Unless the homogeneity of the inoculum is assured, a rapidly growing minority component (*a*) of the population may be responsible for the detectable reactions under one set of conditions (A), whereas the majority component (*b*) of the tested population may propagate under a different set of conditions (B) and manifest its reaction. It is clear that such differences would be difficult to detect if a single criterion such as growth is used as sole indicator of metabolic competence. The ultimate conclusion that a given type may be capable of growth under conditions A and B may be entirely erroneous, since it was type *a* that was responsible for reactions noted under condition A, whereas a completely different type, *b*, accounted for the reactions observed under condition B.

Problems arising during cultural growth

The problems just described apply equally when cultural heterogeneity is not present in the inoculum but develops during subsequent growth. Let us refer again to the last cited example in which nutritional competence under conditions A and B is to be tested, but this time in a population consisting of type *a* cells only. If, as customary in most metabolic studies, the development of turbidity during a given period of growth is used as indicator, one might find equal increase in turbidity under both conditions A and B. Yet a more careful analysis of the final population may reveal that *a* cells grew under condition A, whereas the progeny of a mutant, *b*, which arose during the initial but limited growth of the *a* population, is responsible for the turbidity that developed under condition B. The initial growth of *a*, sufficient to permit the occurrence of mutant *b*, may have been due to previously discussed "carry-over"

effects which permit cells to propagate initially in an adverse environment at the expense of stored products. Such cases have been observed and they show that a careful analysis of the final population should be made in order to avoid what would have been an invalid conclusion in this case, namely that one given type is capable of growing as well under condition A as under condition B.

Similar erroneous conclusions can be drawn if turbidity is used as sole indicator of nutritional competence and growth is observed to occur after a prolonged lag period. The problem involved in such cases, namely the occurrence of a competent mutant during "invisible" growth and the subsequent development of turbidity due to growth of the mutant's progeny, has been clearly demonstrated in the studies of Fildes previously reviewed (p. 58).

In all these cases the occurrence of population changes due to the selective growth of a mutant is much more likely to be detected if viable cell counts (plate counts) are employed. Therefore, it is unwise in any bacteriological study to neglect viable counts. In order to recognize what really happens within bacterial populations it is necessary to employ viable counts, and preferably both viable and total cell counts should be obtained, at least whenever unfavorable cultural conditions exist at the start of the culture.

Since selection pressure does not manifest itself significantly prior to the stationary phase in cultures grown under conditions adequate for the growth of the initially inoculated type, population changes are not too likely to occur during the initial growth of cultures inoculated with a homogeneous population. But whenever such cultures are incubated beyond the point of cessation of logarithmic growth, the possibility of increasing cultural heterogeneity must be recognized.

Primary isolations

The existence of selective host factors favoring the establishment and growth of certain mutants *in vivo* can account for population changes that may occur rapidly during the propagation of isolates under laboratory conditions.[11] There may be not only a lack of the selective host factors *in vitro*, but also altered cultural conditions permitting the accumulation of inhibitory metabolites which favor the rapid establishment of mutants dissimilar to the type which was best adapted to conditions *in vivo*. Therefore, special care must be taken in isolation procedures to insure the propagation of the type

or types present *in vivo*. Whenever feasible, primary isolates should be cultivated in agar media rather than in liquid media, since population changes are generally less apt to occur on solid media than in liquids, provided unduly prolonged incubation is avoided. However, sometimes it is difficult to obtain a positive culture upon first inoculation onto solid media and initial cultivation in liquids becomes necessary. Under such conditions transfer to solid media should be made as soon as feasible in order to avoid drastic population changes. For example, it is customary to make primary isolations of many pathogens in blood cultures. In this case the organisms are propagating in the presence of antiserum, a condition known to support rapid population changes in liquids. In comparison, the presence of antiserum is far less effective in solid media. Unless the likelihood of such population changes during primary isolations is recognized, false conclusions may result regarding the identity of the type present *in vivo*.

CHAPTER 10

Population Changes That Are Not Due to Mutation and Selection

MODIFICATIONS

NOT ALL POPULATION CHANGES IN BACTERIA INVOLVE MUTATION and selection. In Chapters 6 and 7 we referred to the occurrence of modifications, i.e., temporary, non-heritable changes of the phenotype under the influence of environmental agents. Usually such modifications affect all cells of identical genotype simultaneously. However, occasionally cells of identical genotype are not equally affected because not all of them are necessarily in a comparable physiological state required for identical response.

Several previously cited examples (e.g., pp. 103, 112, and 116) illustrate that after transfer to certain environmental conditions all cells in a population may alter their characteristics and maintain these modified characteristics as long as they are kept under these particular conditions. Sometimes the altered phenotype may even be maintained for short periods after return of the cells to the original environment, an effect that may involve intracellular storage of intermediate products as discussed under "carry-over" effects (p. 162). In contrast to population changes due to mutation and selection, where repeated sampling of growing cultures will reveal the gradual progressive establishment of the progeny of favored mutants, modifications will produce simultaneous changes in all or the majority of members of a population. There can be an exception to this over-all population change; it occurs when a population consists of cells with different genotype, all of which display one phenotype under one environmental condition but show differences in detectable characteristics under altered environmental conditions. For example, genetically non-identical cells *a* and *b* may give rise to colonies of similar appearance on medium A but develop into distinctly different colonial types on medium B (Fig. 54). Such

an apparent population change, detectable on medium B, is obviously not due to mutation and selection following transfer from medium A to medium B. This can be confirmed by noting the immediate reversion to phenotypic homogeneity upon transfer to condition A or by isolation of the two types from medium B and subsequent study of the changes in phenotype of the population's components during altered environmental conditions (Fig. 54). Another case of modification that may lead to erroneous conclu-

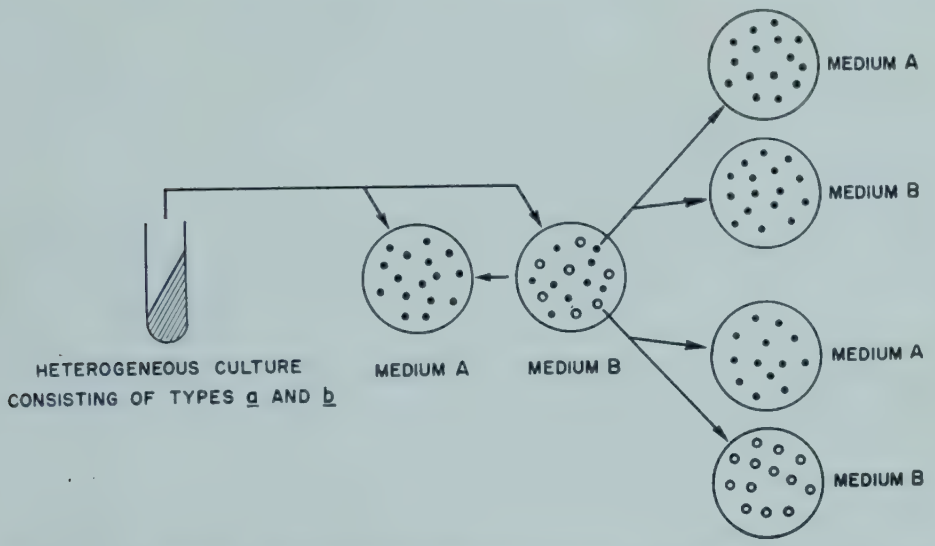


Fig. 54. Diagram illustrating apparent population changes which may be caused by the phenotypic similarity of two genetically different types, *a* and *b*, when grown on one medium (*A*), compared with their dissimilarity on another medium (*B*).

sions regarding the appearance of a new type involves the occasionally observed ability of one mutant in a heterogeneous population to modify the phenotype of another mutant present. Thus, cells giving rise to one distinct colonial type may give rise to a completely different looking type of colony when grown in the presence of certain mutants (Fig. 55). This modification is of the same order as the previously described ability of certain prototrophs to "feed" auxotrophs when they are jointly inoculated into a minimal medium (p. 97). Again, the true situation can be detected by isolating the two distinct components of the population and observing their characteristics when they are grown independently of each other (Fig. 55).

Under the subject of modifications also belong the as yet poorly understood cases of temporary increase in resistance which have

been claimed to occur in some cells after exposure of a population to certain inhibitory agents. Some of these cases have been referred to on page 87. Others have been described by Hinshelwood,[1] who is

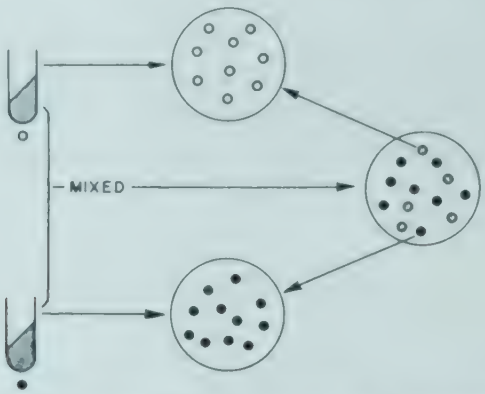


Fig. 55. Diagram illustrating the phenotypic modification of colonial morphology which may occur occasionally when one type is grown in the presence of another colonial type.

the proponent of the widespread occurrence of such modifications. Only a small group of investigators share this belief.

ENZYMATIC ADAPTATION

One special and widely investigated case of temporary modifications in the characteristics of a population is that of enzymatic adaptation. Some general aspects of this phenomenon, which involves the activation of specific enzymatic potentials of a cell under the influence of specific extracellular substrates without changes in genotype, have been reviewed in Chapter 1. We shall now discuss some of the manifestations and applications of enzymatic adaptation in bacteria.[2]

Manifestations and detection

When an unadapted clone of bacteria is brought into contact with an adaptively attacked substrate, e.g., galactoside in the case of certain *E. coli* strains,[3] the overwhelming majority of cells will manifest, after a brief but variable lag period, an increased ability to metabolize the substrate by showing increased β-D-galactosidase

[1] Hinshelwood, C. N., *The Chemical Kinetics of the Bacterial Cell*, Oxford Univ. Press, London, 1946.

[2] See Stanier, R. Y., Ann. Rev. Microbiol., 5:35, 1951, for references.

[3] Lederberg, J., J. Bact., *60*:381, 1950.

activity. Unadapted cells do not necessarily lack the specific enzyme entirely, but the rate of its activity can be so low that it remains undetected by the usual manometric measurements, a fact that makes former distinctions between adaptive and constitutive enzymes merely an artificial distinction. All such enzymatic adaptations require the presence of specific genes controlling the cell's potential capacity to activate specific enzymes under the influence of the proper environment. When a population contains a certain proportion of such competent cells it may appear phenotypically homogeneous under one set of environmental conditions, i.e., in the absence of the specific substrate. After such a population is brought into contact with the appropriate substrate the phenotype of all competent cells will change, revealing the existent genetic heterogeneity. Thus in the process of enzymatic adaptation the detectable characteristics of certain members of a population will be changed even though their genetic constitution remains unaltered. How can one differentiate such population changes, due to enzymatic adaptation of genetically competent cells, from population changes that are due to mutation and selection? First, enzymatic adaptation frequently can occur in the absence of growth, under conditions where selection is entirely eliminated. Thus the exposure of a resting population to the substrate and observation of the subsequent occurrence of the specific metabolic activity constitutes unequivocal proof of the occurrence of enzymatic adaptation. Second, in cases where enzymatic adaptation occurs only in the presence of growth, a condition which makes it difficult to eliminate selection as a causative factor, presumptive evidence against the involvement of mutation can be obtained by the application of the fluctuation test of Luria and Delbrück (p. 52). If the variance obtained in this test is high, mutation must be suspected; if it is low, enzymatic adaptation is indicated. In addition, a single subculture in the absence of the specific substrate usually suffices to de-adapt cells.

Genetic control of enzymatic adaptation

The fact that the potential ability for enzymatic adaptation is under genetic control is illustrated by such studies as those of Monod and Andereau[4] who showed that the capacity for lactose utilization in an *E. coli-mutabile* strain was dependent on the primary occur-

[4] Monod, J. and Andereau, A., Ann. Inst. Pasteur, *72*:868, 1946.

rence of a mutation which endowed the cells with the ability to produce the specific adaptive enzyme. Similar data were obtained for glucose utilization by *P. putrefaciens*.[5] The genetic control of adaptive enzyme formation was further confirmed in Lederberg's recombination experiments with *E. coli* (p. 190), which included studies on the adaptive characteristics of lactose and maltose fermentation.[6]

Simultaneous adaptation

All available evidence indicates that *specific* genes control the potential for the adaptive production of *specific* enzymes. No specific gene change endowing the mutant with the ability to attack a group of *non-related* substrates simultaneously by adaptive enzyme formation has been encountered. Yet bacteria that respond adaptively to a single substrate can thereby produce intermediates which in turn can serve as substrates for the adaptive formation of another enzyme. Here the provision of one external substrate can initiate a kind of chain reaction, causing successive adaptation to a number of *related* substrates each of which is formed by the previous action of an adaptive enzyme. Stanier[7] has termed this phenomenon "simultaneous adaptation" and pioneered its use in the determination of the nature of the intermediates involved in adaptively controlled metabolic pathways. To illustrate the principle of this method a metabolic pathway requiring catalysis by a series of specific adaptive enzymes may be represented as follows:

$$A \xrightarrow[E_A]{} B \xrightarrow[E_B]{} C \xrightarrow[E_C]{} D \xrightarrow[E_D]{} \text{etc.}$$

where E_A, E_B, etc. represent the specific adaptive enzymes catalyzing each step. These enzymes are not apparent unless the cells are exposed to specific substrates, A, B, etc. Exposure to substrate or condition A will cause the production of E_A, thereby catalyzing step $A \longrightarrow B$. The formation of B will in turn stimulate the activity of E_B, causing the formation of C, etc. Therefore, cells adapted to metabolite A will simultaneously also be adapted to metabolites B, C, D, etc. Similarly, the initial adaptation of cells to any intermediate in the pathway will cause the full adaptation to later inter-

[5] Klein, H. P. and Doudoroff, M., J. Bact., *59*:739, 1950.

[6] Lederberg, J., Lederberg, E. M., Zinder, N. D. and Lively, E. R., Cold Spring Harbor Symp. Quant. Biol., *16*:413, 1951.

[7] Stanier, R. Y., J. Bact., *54*:339, 1947.

mediates in the same reaction chain. In view of its specificity this phenomenon permits testing whether a presumed intermediate actually belongs in a given reaction chain consisting of a series of steps which involve enzymatic adaptation. In the actual tests an organism is adapted to a primary substrate and is then tested for its ability to dissimilate the presumed intermediate. If the compound under investigation is immediately attacked by the cells adapted to the primary substrate A, but is not similarly dissimilated by control cells unadapted to A, then it is highly probable that the compound is a member of the reaction chain. Metabolic processes of bacteria which have been analyzed, partly or entirely, by an analysis of adaptive patterns include oxidation of acetate and Krebs-cycle compounds, oxidation of aromatic compounds, oxidation of tryptophan and of tyrosine, and the fermentation of purines.[2]

Kinetics of enzymatic adaptation

Much has been learned about the kinetics of enzymatic adaptation (see p. 27) in bacteria, and some interesting data have been obtained about substrate interactions which can occur with certain adaptively attacked compounds and are indicative of competitive interactions in the synthesis of different specific enzymes.[8] For example, *B. subtilis* cells grown in the presence of D-fructose and L-arabinose display two successive growth cycles ("diauxie"), separated by a period of lag or even decrease (Fig. 56). According to Monod, each of these cycles corresponds to the exclusive utilization of one compound at a time; the second compound is not attacked until the first has been exhausted. Experimental data indicate that this is due to an inhibition by the first compound upon the formation of the adaptive enzyme attacking the second compound. Manifestation of this competition, i.e., the resulting growth curves (Fig. 56), shows a striking resemblance to the successive cycles of increase in viable cell counts obtained in studies on population changes due to mutation and selection (Fig. 49), even though the time element involved differs drastically between the two phenomena. Thus the kinetics of *intercellular* competition (mutation and selection) resemble those of *intracellular* competition (enzymatic adaptation), lending support to the assumption that autocatalytic (self-propagating) cytoplasmic elements may be involved in the formation of adaptive enzymes (p. 27).

[8] Monod, J., Growth Symposium, *11*:223, 1947.

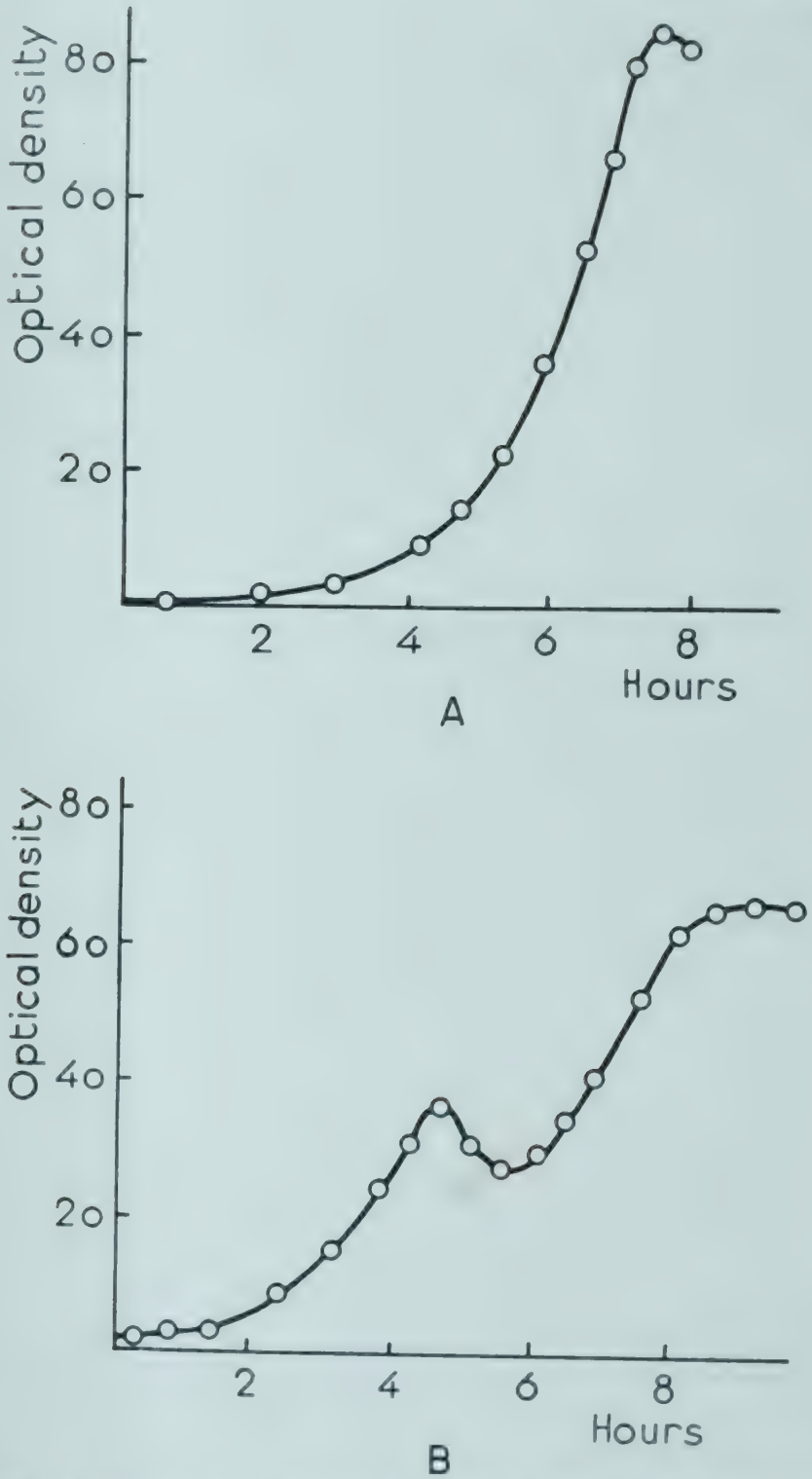


Fig. 56. *A*, Growth of *B. subtilis* in synthetic medium with saccharose + D-mannose as carbon source, normal growth curve; *B*, growth of *B. subtilis* in synthetic medium with D-fructose + L-arabinose as carbon source, "diauxic" curve. (From Monod.[8])

Finally, recent evidence[2] indicates that the continuous presence of the specific substrate is not always a prerequisite for adaptive enzyme formation but that in certain cases, e.g., penicillinase formation by *Bacillus cereus,* a fleeting exposure to the substrate may suffice to cause subsequent temporary high enzyme activity in the absence of the substrate. But even though this as yet rarely encountered phenomenon permits the continued maintenance of an altered phenotype after transfer to new environmental conditions, it can be distinguished from population changes due to mutation and selection by the fact that it involves a simultaneous change of all competent cells and fails to persist for more than a few generations.

CHAPTER 11

Recombination in Bacteria

THE MAJOR SOURCES OF GENETIC VARIABILITY IN SEXUALLY REproducing higher organisms are mutation and recombination. The latter is due to the reassortment of non-identical chromosomes or chromosomal components, half of which were contributed by the father and half by the mother. In Chapter 1 random segregation of each member of a chromosomal pair during the maturation division was described. As illustrated in Figure 7, this reassortment of chromosomes can cause new genotypes by bringing together new interchromosomal combinations in the gametes. However, all genes located on one chromosome, or, as it is usually expressed, genes belonging to the same linkage group, will remain together during this chromosomal segregation. Yet even genes located on the same chromosome can be reassorted by a process known as *crossing-over*. This particular process of recombination involving the exchange of chromosome parts between the two members of the same chromosome pair has been confirmed in higher organisms both by breeding experiments and by cytological observations.

MECHANISM OF CROSSING-OVER IN HIGHER ORGANISMS

Briefly, and somewhat simplified, this is the manner in which this exchange of parts of the chromosome can take place (Fig. 57). Prior to segregation of the members of each chromosome pair the two chromosomes are in intimate contact. At this time corresponding parts of each chromosome can be exchanged in a process that presumably involves breakage and immediate fusion. As Figure 57 illustrates, only two of the four chromatids in each pair of chromosomes are involved in this exchange; consequently, two of the four gametes resulting at the end of meiosis (see p. 7) will contain chromosomes with the recombination of genes brought about by the crossing-over of chromatids. If one member of a chromosome pair carries genes A B C D E F and the other member carries genes

a b c d e f, crossing-over between the sites of genes B and C or b and c will result in the creation of new chromosomal gene-combinations, namely A B c d e f, and a b C D E F. The resulting gamete transmits the new gene-arrangement to succeeding generations where the previous occurrence of the crossing-over can be recognized by the novel phenotype corresponding to the novel genotypic intrachromosomal assortment. Since cross-overs are more likely to

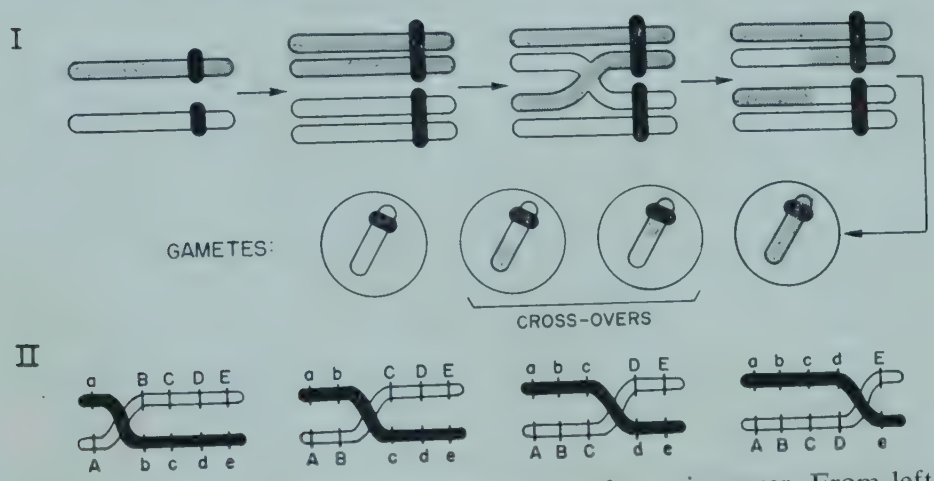


Fig. 57. *I*, Diagrams illustrating the process of crossing over. From left to right: Two homologous chromosomes before meiosis; each chromosome becomes double as it forms two chromatids; one chromatid from each chromosome exchanges a portion with the homologous chromatid of the other chromosome; crossing over is completed, two of the four gametes resulting at the end of meiosis carry cross over chromosomes. *II*, Diagrams illustrating the likelihood of occurrence of crossing over among 5 gene pairs located at different sites of a chromosome pair. The distant genes A and E are separated from each other by all of the possible crossing overs, the likelihood of crossing over between A and E equalling the sum of the occurrence of crossing over between genes A and B, B and C, C and D, D and E. Similarly, the probability of crossing over occurring between genes A and C is greater than that between genes A and B.

occur between genes that are located far apart it has been possible to establish the relative position of genes within a chromosome on the basis of cross-over frequencies. As shown in the second part of Figure 57, crossing-over will occur more frequently between distant genes A and E than between neighboring genes A and B. Morgan, who did the pioneer work on these problems with the fruit fly in 1915, found that the analysis of cross-over data from breeding experiments permitted the construction of *linkage maps* for individual chromosomes on which genes were located in a linear fashion ac-

cording to the distance indicated by "cross-over units." For example, mutant genes a, b, and c giving rise to such easily detected phenotypes as cinnabar eye color, short wings, and curved wings were known to belong to the same linkage group, i.e., were located on the same chromosome, because they usually segregated together in crosses with the wild type. Yet the appropriate breeding experiments revealed that occasionally genes a, b, and c may separate from each other and this separation occurs with a constant frequency. Ten per cent of the progeny showed the occurrence of segregation between characteristics a and b, and 18 per cent between a and c. Accordingly, on the linkage map genes a and b are plotted at a distance of 10 units from each other, and genes a and c are separated 18 units from each other. In studies with many plants and animals the validity of such linkage maps, constructed with the aid of breeding experiments involving a great variety of mutants, was confirmed, and in the case of the fruit fly this was further substantiated by cytological observations on the giant salivary gland chromosomes.

Recombination in *E. coli*, K-12

Obviously, such phenomena are only possible among sexually reproducing organisms in which parents with different genotype can contribute the non-identical chromosomal complements to their offspring and thereby permit a subsequent recombination of the genes and the characteristics controlled by them. Since the existence of a sexual process among bacteria always had been an open question, several investigators, principally Browning,[1] Sherman and Wing[2] and Gowen and Lincoln,[3] made some inconclusive attempts to detect a recombination of characteristics following mixed cultivation of mutant strains of *E. coli*, *Aerobacter aerogenes*, and *Phytomonas stewartii*. The first conclusive results in this direction were obtained in the studies conducted by Tatum and Lederberg[4] on mutant strains of *E. coli*, K-12. These investigators used auxotrophic parent strains, unable to grow on minimal medium, and recognized the occurrence of recombination by isolating, following mixed cultivation of these strains, cells which were able to grow

[1] Browning, C. H., J. Path. Bact., *12*:166, 1908.
[2] Sherman, J. M. and Wing, H. U., J. Bact., *33*:315, 1937.
[3] Gowen, J. W. and Lincoln, R. E., J. Bact., *44*:551, 1942.
[4] Tatum, E. L. and Lederberg, J., J. Bact., *53*:673, 1947.

on minimal medium and displayed characteristics of both parent types (Fig. 58). Thus one parent strain (58-161) required biotin (B^-) and methionine (M^-) for growth, the other parent strain (W-1177) required threonine (T^-), leucine (L^-) and thiamine (B_1^-).[5] In addition the latter strain carried the following "markers": Lac^- Mal^- Mtl^- Xyl^- Gal^- Ara^- (non-fermenter of lactose, maltose, mannitol, xylose, galactose, and arabinose), as well as phage-resistance (V_1^r) and streptomycin-resistance (S^r). These strains

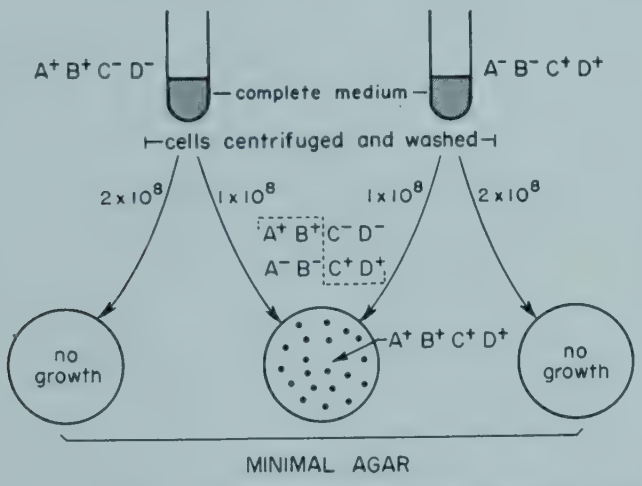


Fig. 58. Occurrence of recombination following mixed cultivation of two auxotrophic strains of *E. coli*, K-12, on minimal agar.

had been derived by a series of individual mutational steps from the original K-12 parent culture. Spontaneous reversion of either auxotroph strain to the prototrophic condition (ability to grow on minimal medium) was never observed. Since such reversions would require simultaneous mutation of two genes in the case of the 58-161 strain, namely B^- and M^-, and simultaneous mutation of three genes in the case of the W-1177 strain, namely T^-, L^-, and B_1^-, such spontaneous changes would not be likely to occur. It should be remembered that spontaneous mutations from a specific auxotrophic type to the prototrophic condition, e.g., B^- to B^+, generally occur at rates of 1×10^{-7}, a simultaneous reversion of a double auxotroph to prototrophy, e.g., B^- M^- to B^+ M^+, would only be likely to occur at rates as low as 1×10^{-14}. However, when the

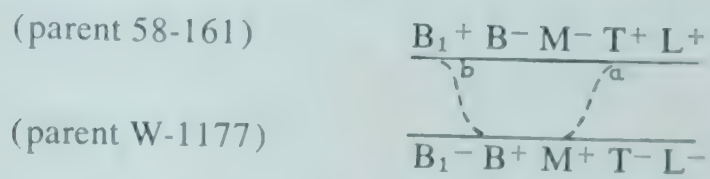
[5] Throughout this chapter the mutant symbols will be cited as they were given in the original publications, but it should be kept in mind that in this instance the use of capital letters does not necessarily reflect the dominance of the genes involved.

above parent strains were plated in mixture on a minimal medium, prototroph colonies developed at the rate of approximately 1 for every 10^7 cells plated. Thus the auxotroph mixture:

$$B^- M^- T^+ L^+ B_1^+ \times B^+ M^+ T^- L^- B_1^-$$

yielded a very small number of stable prototroph cells of the genotype $B^+ M^+ T^+ L^+ B_1^+$.

This result was explained most easily on the assumption that sexual fusion of certain parent cells and subsequent segregation of genes in new combinations may occur. In other words, it was assumed that two cells, one from parent strain *A*, the other from parent strain *B*, may mate, forming a zygote from which parental and recombinant cells may emerge following reduction division. Subsequent studies supported this assumption. Thus, it was demonstrated that all conceivable combinations of the characteristics of both parents can be detected among the "recombinants." For example, if mixed platings were made on a minimal medium enriched with thiamine, $B^+ M^+ T^+ L^+ B_1^-$ cells could develop into colonies and were found to occur in addition to the $B^+ M^+ T^+ L^+ B_1^+$ prototrophs after mixed plating of the parent strains. However, the proportion of these B_1- recombinants recoverable from mixed platings of a given number of parent cells was always greater than the proportion of recoverable $B^+ M^+ T^+ L^+ B_1^+$ prototrophs. This indicated that segregation did not take place arbitrarily but that certain forces controlled the likelihood of genes introduced with one of the parents to separate from their original combination and to recombine and segregate with the genic complement introduced by the other parent. Linkage groups similar to those discovered in studies with higher organisms appeared to exist. Thus if the genes for the tested characteristics were arranged in one chromosome as follows:



a single cross-over between M and T (at site a) may occur relatively frequently in cells after sexual fusion, yielding $B_1^- B^+ M^+ T^+ L^+$ recombinants after segregation, whereas a recombinant cell of the type $B_1^+ B^+ M^- T^+ L^+$ would require the occurrence of a

double cross-over (at sites a and b), an event which is less likely to occur than a single cross-over. In this manner the linkage relationships of various genes were tested by inspection of the frequency with which various recombinations occurred. The results were in general agreement with the idea that the tested genes of *E. coli*,

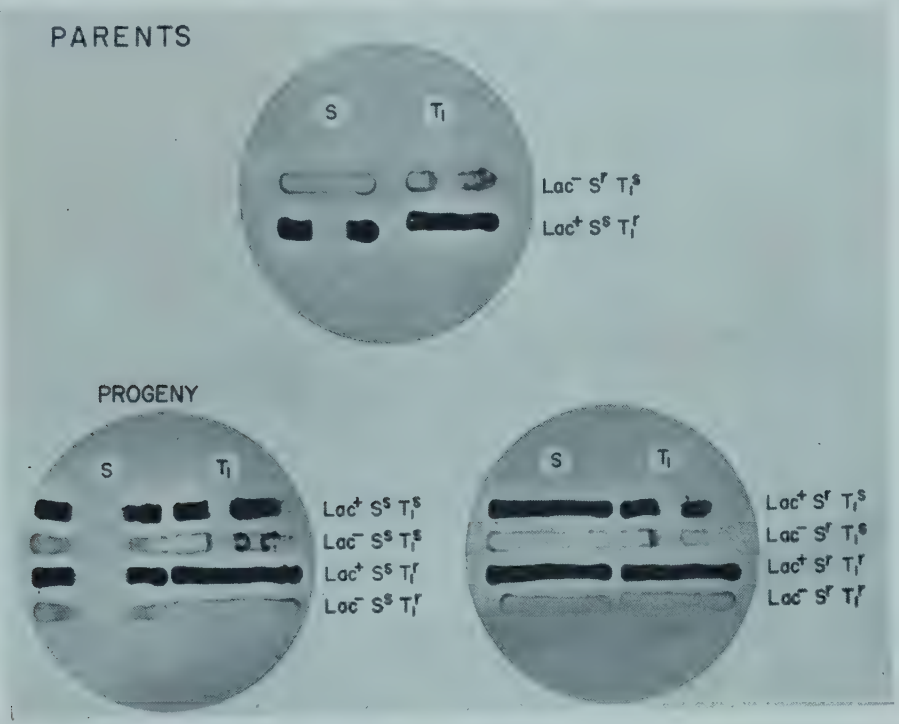


Fig. 59. Recombination among unselected markers in an experiment with strains derived from *E. coli*, K-12. Samples from the auxotrophic parent strains and from recombinant, prototrophic colonies have been streaked on EMB-lactose agar to detect capacity for lactose fermentation; they also were cross-streaked with a solution of streptomycin (S) and a suspension of phage (T_1) to determine streptomycin- and phage-resistance. A break in the streak in the area of cross-streaking is indicative of susceptibility to phage or streptomycin, rèspectively. (H. B. Newcombe.)

K-12, are arranged linearly at definite distances from each other in one linkage group (chromosome) and that the observed recombinations occurred by sexual fusion and recombination between haploid parent cells.[6,7,8]

These conclusions were strengthened by studies on the "unselected" markers such as fermentation characteristics and phage- or

[6] Lederberg, J., Genetics, *32*:505, 1947.
[7] Lederberg, J., Lederberg, E. M., Zinder, N. D. and Lively, E. R., Cold Spring Harbor Symp. Quant. Biol., *16*:413, 1951.
[8] Rothfels, K. H., Genetics, *37*:297, 1952.

antibiotic-resistance. The latter characteristics are termed "unselected" markers because they may show arbitrary, non-selected recombinations in the prototroph cells isolated following crosses made between multiple auxotroph parents on minimal media, where the growth requirements serve as "selected" markers permitting the rapid identification of recombinants which are represented by all those cells capable of developing into colonies on minimal media (Fig. 59). Thus mixed plating on minimal media of

	selected markers	unselected markers
(parent 58-161)	B_1^+ B^- M^- T^+ L^+	Lac^+ Mal^+ Mtl^+ Xyl^+ Gal^+ Ara^+ V_1^s S^s
(parent W-1177)	B_1^- B^+ M^+ T^- L^-	Lac^- Mal^- Mtl^- Xyl^- Gal^- Ara^- V_1^r S^r

can give rise to all of the 2^6 possible combinations (256) among the unselected markers if a sufficient number of recombinant colonies are examined. For example:

B_1^+ B^+ M^+ T^+ L^+	Lac^+ Mal^- Mtl^- Xyl^+ Gal^+ Ara^+ V_1^s S^s
B_1^- B^+ M^+ T^+ L^+	Lac^- Mal^+ Mtl^+ Xyl^- Gal^- Ara^+ V_1^r S^r
B_1^+ B^+ M^+ T^+ L^+	Lac^- Mal^- Mtl^+ Xyl^+ Gal^- Ara^+ V_1^r S^s
B_1^+ B^+ M^+ T^+ L^+	Lac^+ Mal^+ Mtl^- Xyl^- Gal^+ Ara^+ V_1^s S^r
	etc.

Again, certain recombinations occur more frequently than others, indicating different degrees of linkage, i.e., distance between different genes on one chromosome, in this case between genes controlling fermentation and resistance characteristics.

Additional support for the existence of a linkage system is provided by the following example: if the segregation were at random, "reversed" crosses should yield identical results, i.e.,

$$B^- M^- T^+ V_1^s \times B^+ M^- T^- V_1^r \qquad (1)$$

should give results identical to those obtainable from

$$B^- M^- T^+ V_1^r \times B^+ M^- T^- V_1^s \qquad (2)$$

where phage-resistance (V_1^r) is introduced with the other parent. Actually, it can be shown that the majority of prototrophs in cross (1) are phage-sensitive (V_1^s), whereas the majority recovered from cross (2) are phage-resistant. This is in agreement with the assumed existence of a linkage group because in the cross

$$\times \quad \frac{B^- \; M^- \; T^+ \; V_1^s}{B^+ \; M^+ \; T^- \; V_1^r}$$

a single cross-over will unite V_1^s with the $^+$ genes whereas a double cross-over is required to unite V_1^r with the $^+$ genes. As stated before the probability for single cross-overs is far greater than that for double cross-overs. In contrast the "reversed" cross

$$\times \quad \frac{B^- \; M^- \; T^+ \; V_1^r}{B^+ \; M^+ \; T^- \; V_1^s}$$

yields a greater proportion of V_1^r recombinants among the prototrophs because now a single cross-over will combine V_1^r with the $^+$ genes, whereas a double cross-over is required for the combination $B^+ \; M^+ \; T^+ \; V_1^s$.

Lederberg[9] has demonstrated that growth-inhibitors also can be used efficiently for selective markers in recombination experiments. Thus mixed platings of a streptomycin-resistant (sr) strain of *E. coli*, K-12, with an azide resistant (azr) K-12 strain on media containing both streptomycin and azide yielded sr-azr recombinants capable of growth into colonies, whereas either parent strain proved incapable of growing on such a medium, except for the rare occurrence of sr mutants among azr cells, or of azr mutants among sr cells. The rate of such spontaneous mutants was found to be 1 per 7×10^9 cells and 7 per 1×10^9 cells, respectively; however, mixed azr and sr cultures yielded an average of 250 dually resistant cells per 10^9 cells plated. The presence of unselected markers (fermentation characteristics) in the parent strains confirmed that 93 per cent of dually resistant cells recovered from plating azr and sr cells on an azide-streptomycin-containing medium represented recombinants. Since the isolation of mutants resistant to growth-inhibitors is usually far simpler than the isolation of nutritional mutants, the use of resistance characteristics as selective markers should prove of value in searching for the occurrence of recombination among other as yet untested bacteria. One precaution required in applying this method is the use of inhibitory agents which do not interact. As an example, Lederberg cites penicillin and iodoacetate, which produce synergistic effects and, therefore, are unsuited for the screening of dually resistant cells.

True recombination versus transformation

In view of the fact that in certain bacterial species, e.g., in pneumococci, filterable substances, diffusing through the medium,

[9] Lederberg, J., J. Bact., *59*:211, 1950.

are capable of "transforming" cells in such a manner that specific, hereditary properties are induced in dissimilar cell types (see Chapter 12), the question arose whether the above data could be explained on the basis of "transforming substances." Lederberg[6] has provided a number of experiments which make this explanation unlikely. For example, neither culture filtrates nor cell autolysates prepared from one of the parent strains are capable of producing the described genetic changes in the other parent strain. In addition, the linkage relationships and the fact that whole groups of marker-genes appear recombined in the progeny argue against the involvement of transforming substances. The most substantial proof against the role of diffusible transforming substances in the recombination phenomenon was provided in an experiment described by Davis.[10] He employed a U-tube containing an ultrafine fritted-glass filter at the base. In such a tube bacteria growing on either side of the filter are prevented from physical contact, yet diffusible substances can pass freely from one side of the tube to the other. When cells of strain 58-161 were inoculated into one arm of the tube, filled with complete broth, and cells of strain W-1177 were inoculated into the other arm, subsequent plating into minimal medium failed to uncover any recombinants. In contrast, when both parent strains were inoculated into the same arm, thus permitting direct contact between the cells, the expected number of recombinants could be recovered.

Mechanism of bacterial recombination

The foregoing data strongly suggest that physical contact, probably cellular fusion, is required for the observed recombination phenomena. Yet so far no one has seen such fusions, an event which is unlikely to be observed since only approximately 1 out of 10^7 cells appears to take part in this process. There have been some suggestions that instead of direct fusion the transfer of genetic properties might occur via phage, a remote possibility that might be supported by the fact that K-12 as well as several other recombining strains (see below) have been found to carry phage, i.e., they are lysogenic. In addition, it has been reported by Hayes[11] that recombination can occur even after multiplication of one of the parents has been inhibited by exposure to streptomycin. At the moment

[10] Davis, B. D., J. Bact., *60*:507, 1950.
[11] Hayes, W., Nature, *169*:118, 1952.

too little is known about the physical basis of recombination to make any informed guesses regarding its actual mechanism. However, it has become evident that even if the transfer of genetic properties is indirect, the particles involved must act as true gametes transferring the total genotypic complement of one parent to the other parent (receptor) cell. This is demonstrated by the occurrence of cells in which the segregation of the genetic complements contributed by the two parents does not occur immediately. Lederberg[12] has found such unique temporarily diploid, heterozygous cells which propagate for a number of generations carrying the genotypes contributed from both parent strains. Thus, if the usual *E. coli* cells possess one chromosome, these diploid cells must possess a pair of chromosomes, as revealed by the genetic analysis of such cells and their progeny.

It has been suspected that the low frequency of recombination, i.e., approximately 1 recombinant cell per 1×10^7 cells plated, may be due to our lack of knowledge regarding some unknown factors which promote the occurrence of recombination. For example, hereditary compatibility factors determining sexual potencies among strains might be involved. However, factors of this type have not been uncovered so far.[12a] On the other hand, a variety of environmental changes may result in *apparently* increased frequencies of recombinations.[13, 14] Thus, in a typical recombination experiment involving nutritional requirements as selective markers the two parent strains are grown separately in a complete medium for 18 hours and aliquots of each culture containing 1×10^8 cells are mixed on the surface of a minimal agar plate. The number of prototroph colonies that develop after 2 to 3 days of incubation will then be found to be in the neighborhood of 20, indicating a rate of recombination of 1 per 1×10^7 cells. However, if cells are passed through a rich nutritional medium prior to plating, e.g., if they are grown in embryonated eggs, as many as 2000 prototroph colonies may develop from the mixed plating of 2×10^8 auxotroph parent cells.[14] This apparent increase in the frequency of recom-

[12] Lederberg, J., Proc. Nat. Acad. Sci., *35*:178, 1949.

[12a] A report describing the existence of a hereditary factor controlling sexual compatibility in *E. coli* (Lederberg, J., Cavalli, L. L. and Lederberg, E. M., Genetics, *37*:720, 1952) appeared while this book was in press.

[13] Haas, F., Wyss, O. and Stone, W. S., Proc. Nat. Acad. Sci., *34*:229, 1948.

[14] Whallon, J. and Braun, W., Bact. Proc., 1952, p. 44.

bination has been shown to be due merely to the increased residual growth of the parent auxotroph cells on the minimal medium. Therefore, if the rate of recombination is calculated not on the basis of the number of cells initially used for plating on minimal medium but is more properly calculated on the basis of the final auxotroph parent population, the rate of recombination again turns out to be 1 per 1 x 10^7 cells.

Prevalence of recombination among different bacteria

All the results described above have been obtained in studies with *E. coli*, K-12, a typical coliform bacterium originally isolated from human feces. Subsequent attempts to detect similar phenomena in other widely used laboratory strains of *E. coli*, such as strain B, met with failure except for the discovery of one additional strain in England[15] that could be crossed with K-12. In view of this apparent rarity of "fertile" strains Lederberg[16] tested a large number of cultures isolated from human urine and chicken cecal flora for their ability to cross with K-12, using streptomycin-resistance and prototrophy as selective markers. Among 100 human isolates he found 8 that cross with the same facility as K-12. One of the 40 chicken isolates examined also crossed with K-12. This finding removed strain K-12 from its position as the only bacterium with a "sexual" mode of reproduction. Subsequently, additional fertile strains were detected, bringing their total to at least 50.[17]

There has been little concerted effort to test recombination in other bacteria along the lines described in the preceding sections. Suggestive, though not entirely conclusive, evidence for recombination has been obtained in studies with auxotrophic mutants of *Achromobacter fischeri*.[18] In contrast, extensive efforts to uncover recombination among *Salmonella* strains proved futile, but have led to the discovery of a different mechanism of intercellular transfer of genetic properties (see Chapter 12).

Problems that require recombination for testing

Genetic recombination furnishes investigators in the field of bacterial genetics with the same type of analytical tool that has

[15] Cavalli, L. L. and Heslot, H., Nature, *164*:1057, 1949.
[16] Lederberg, J., Science, *14*:68, 1951.
[17] Lederberg, J., personal communication.
[18] McElroy, W. D. and Friedman, S., J. Bact., *62*:124, 1951.

proved its unique usefulness in genetic studies with higher organisms.[19] Above all it provides an opportunity to demonstrate critically the genetic control of characteristics subject to variation and to prove the mutational nature of bacterial variants. Provided that recombination involves the exchange between chromosome-like structures, as seems to be the case, it also should provide means of distinguishing whether a given characteristic is controlled by nuclear or cytoplasmic factors (see p. 126). In addition, the linkage relationships that can be detected by inspection of the recombinants permit an analysis of the relationship between genetic factors controlling a series of related characteristics. Thus the relationship between various streptomycin-resistant and streptomycin-dependent mutants derived from *E. coli,* K-12, was tested in crosses with the sensitive type and with each other.[20] The results showed that resistance and dependence are inherited as if they were controlled by the same gene locus. No crossing-over occurred between the factors controlling these properties. Such a phenomenon involving control of a series of related characteristics, usually differing quantitatively, by what appear to be alternative changes occurring at the same locus in a chromosome *(multiple alleles)* is well known in higher organisms. Several sensitive and resistant mutants derived from a streptomycin-dependent mutant also seemed to belong to this series of multiple alleles. Another problem that became analyzable with the help of recombination, utilizing those unique diploid strains in which segregation following fusion was delayed, concerns the relationship between a mutant gene and its normal allele. For example, crosses between a streptomycin-sensitive (+) and a streptomycin-resistant (sr) parent strain led to a number of diploid heterozygotes which carry the $\frac{sr}{+}$ factor from one parent and the sr factor from the other parent. These $^{sr}/_{+}$ cells proved to be susceptible to streptomycin, demonstrating that streptomycin-sensitivity is fully dominant to streptomycin-resistance, the latter being the recessive factor.[21] Similarly Lac+was found to be dominant to Lac$^-$ and phage-sensitivity proved to be dominant to phage-resistance.[12] Since most bacteria are generally regarded as haploid, such domi-

[19] See Lederberg, J. *et al.,* Cold Spring Harbor Symp. Quant. Biol., *16:*413, 1951, for a detailed description of studies in which bacterial recombination has been used for the analysis of various genetic problems.

[20] Newcombe, H. B. and Nyholm, M. H., Genetics, *35:*603, 1950.

[21] Lederberg, J., J. Bact., *61:*549, 1951.

nance relationships attain little importance as far as the development of bacterial resistance is concerned. However, increasing evidence has accumulated indicating that many bacteria are multinucleate, at least during certain stages of a culture's growth. A mutation to resistance, such as sr, occurring in one nucleus would then be unable to manifest itself phenotypically since one or more "+" nuclei present would exert their dominance effect over the sr gene. The streptomycin characteristic would not manifest itself until subsequent fissions led to either uninucleate cells containing only the sr nucleus or multinucleate cells containing sister nuclei, all carrying the sr factor.[20] Dominance in multinucleate cells and subsequent nuclear segregation thus can account at least in part for the frequently observed phenotypic lag in bacteria (see p. 75). Finally, genetic recombination, if proved to be relatively frequent among bacteria, may provide a source for the appearance of recombinant strains with uniquely reshuffled characteristics which may play a role both in epidemics and bacterial evolution. We shall return to a consideration of such problems in Chapter 13.

CHAPTER 12

Bacterial Transformation and Transduction

TRANSFORMATION

In the preceding sections we have stressed repeatedly that both spontaneous and induced changes in the genetic properties of organisms are characterized by the fact that they are entirely undirected and independent of the existing environmental conditions. One of the long-standing but unachieved ambitions of geneticists has been to detect means whereby specific mutations can be induced at will. This ambition seemed closer to realization when bacteriologists demonstrated that in a few bacterial species specific inheritable changes can be induced under the influence of desoxyribose nucleic acid fractions isolated from genetically dissimilar members of the same species. Since the nuclear determinants of hereditary characteristics in higher organisms have been identified with desoxyribose nucleic acid, the so-called "transformation" discovered among bacteria has been recognized as a phenomenon of basic biological significance which promises to be of material assistance in elucidating the specificity and mode of action of genetic determinants.

Definition of transformation

Transformation reactions permit certain strains of bacteria, grown in the presence of killed cells, culture filtrates or extracts from other related strains, to acquire, and to manifest in subsequent generations, certain properties of the related strain. They have been defined as a hereditary alteration in a susceptible cell resulting from the acquisition from its environment, by other than cellular fusion, of a genetically active unit directing the inheritable change. Following such reactions, the genetically active material is demon-

strable in the progeny of the transformed cell, grown in the absence of the transforming factors, and is recoverable from such populations in excess of the amount required to induce the initial alteration, i.e., the transforming principle behaves as a self-reproducing substance.[1] So far transformations have been described in only a few species, but since specific environmental conditions are often necessary for these reactions, the scarcity of their demonstration may be a reflection of lack of proper techniques rather than of a lack of potential to react.

Transformation of pneumococcal capsular types

Transformation was observed first with certain strains of *Pneumococcus* and most of the subsequent work on the mechanism of transformation has been carried out with this species. The so-called virulent smooth (S) type of *Pneumococcus* possesses a capsule that is composed essentially of polysaccharide.[2] The chemical composition and immunological properties of capsular polysaccharide show specific differences among different strains. Almost 100 different serological types have been detected among smooth pneumococci. This serological type specificity is a hereditary property and is maintained during prolonged cultivation as long as the cells remain smooth. Spontaneous mutation from one type-specific S type to another capsular type has never been observed. However, in a fashion similar to the spontaneous changes in antigenicity and colonial morphology described for other bacterial species (Chapter 7), S strains may undergo population changes involving the establishment of spontaneous R mutants. The R type cells are nonencapsulated, lack the serological type specificity of their S parent cells, and give rise to colonies that are more granular than those

[1] Austrian, R., Bact. Rev., *16*:31, 1952.

[2] The smooth-rough terminology introduced by Griffith for pneumococcal variants differing in antigenicity and colonial morphology does not correspond to similar terms applied to analogous variants in most other bacterial species. Thus the Griffith S corresponds to variants commonly designated as mucoid (M) in other species, rough (R) pneumococci correspond to variants usually designated as S, "extremely rough" (ER) pneumococcal variants (Taylor[13]) correspond to types designated R in most other species, and the intermediate (I) variants of pneumococci appear comparable to "weakly M" types of other species. The Griffith terminology has been retained by those interested in the study of *Pneumococcus* and appropriate changes in terminology have been discouraged in order to avoid added confusion.

formed by S cells. Contrary to statements in the older literature, non-encapsulated R cells apparently never revert spontaneously to any encapsulated types[1] (see also footnote 14).

In 1928 Griffith[3] discovered that it was possible to transform a non-encapsulated R variant derived from one specific type into encapsulated S cells of a heterologous type by *in vivo* passage in the presence of heat-killed heterologous S cells. He injected a small number of live R cells into mice simultaneously with a large number of heat-killed S cells with serological specificity different from that of the S strain from which the R cells had been derived. Many of these mice died subsequently and pneumococci isolated from them were found to be virulent, living S cells displaying the same type specificity as that of the killed S cells inoculated. Thus:

$$\text{S II} \rightarrow \text{R} \xrightarrow[+\ \text{heat-killed S III}]{\text{into mice}} \text{S III}.$$

Several investigators successfully repeated this experiment and eliminated the possibility that the results may have been due to contamination of the inoculum with a few live S III cells. In much later work the possibility of accidental contamination was ruled out even more conclusively by employing R cells "labelled" with a specific somatic protein antigen or with drug-resistance,[4, 5] properties which vary independently of capsular polysaccharide. Thus:

$$\text{S II (M2')} \rightarrow \text{R(M2')} \xrightarrow[+\ \text{heat-killed S III}]{\text{into mice}} \text{S III (M2')}.$$

(specific M protein)

In 1931 Dawson and Sia[6] obtained similar transformations *in vitro* by growing an R strain in broth containing anti-R serum and heat-killed S cells. Subsequently, Alloway[7] succeeded in obtaining specific *in vitro* transformations in the presence of cell-free extracts obtained from S cells. This opened the way for the brilliant investigations of Avery, MacLeod and McCarty,[8] who succeeded in isolat-

[3] Griffith, F., J. Hyg., *27*:113, 1928.
[4] Austrian, R. and MacLeod, C. M., J. Exp. Med., *89*:451, 1949.
[5] Langvad-Nielsen, A., Acta Path. Microbiol. Scand., *21*:362, 1944.
[6] Dawson, M. H. and Sia, R. H. P., J. Exp. Med.. *54*:681, 1931.
[7] Alloway, J. L., J. Exp. Med., *55*:91, 1932; *57*:265, 1933.
[8] Avery, O. T., MacLeod, C. M. and McCarty, M., J. Exp. Med., *79*:137, 1944.

ing the active transforming principle (TP) in the form of desoxyribonucleic acid (DNA). Crude transforming extracts obtained from heat-killed encapsulated type III cells proved to contain many inactive components, including protein, lipids, ribonucleic acid, and polysaccharides. When these were removed by chloroform extraction, enzymatic hydrolysis and alcohol fractionation, a final product was obtained which possessed practically all the activity of the crude extract. By a variety of means, including inactivation

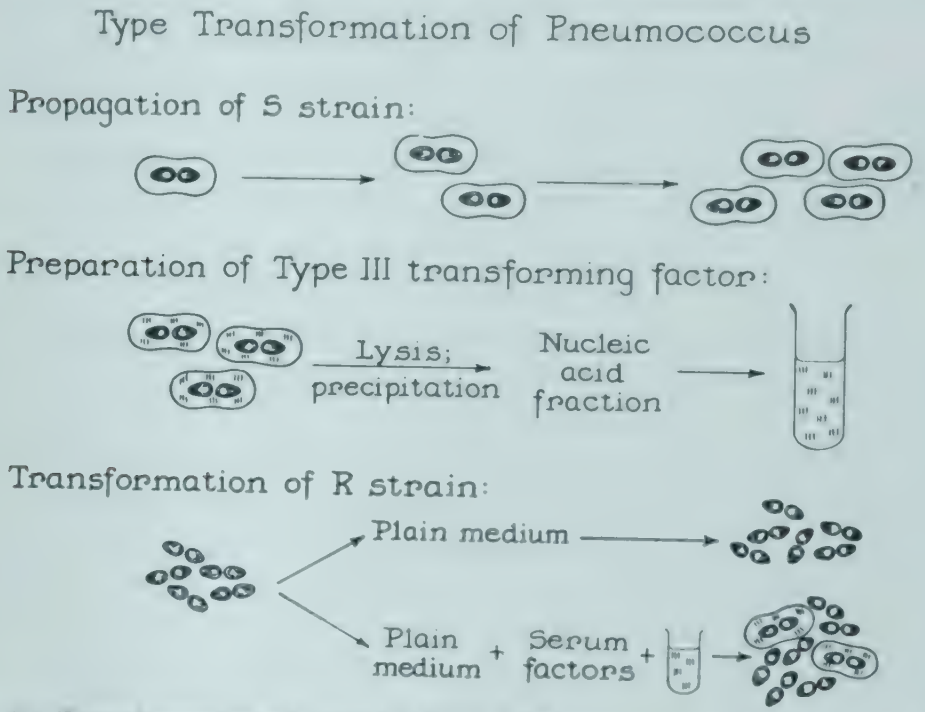


Fig. 60. Transformation of capsular type in *Pneumococcus*. (R. Hotchkiss.)

by purified desoxyribonuclease, chemical analysis, and ultraviolet absorption curves the DNA nature of the TP was proven.[9] Additional studies[10] gave convincing proof that the active fraction owes its activity merely to highly polymerized DNA; there is no reason to believe that it is contaminated with traces of possibly active protein, and it is free of polysaccharide. The purified DNA is highly active, as little as 1 part in 600,000,000 is capable of causing specific transformation of competent R cells in appropriate media (Fig. 60). Thus:

$$\text{S II} \rightarrow \text{R} + (\text{DNA from S III}) \rightarrow \text{S III}.$$

[9] McCarty, M. and Avery, O. T., J. Exp. Med., *83*:89, 1946; *83*:97, 1946.

[10] Hotchkiss, R. D., Colloques sur "Les unités biologiques douées de continuité génétique." Centre Nat. Rech. Sci., Paris, *8*:57, 1948.

Not only is the induced change permanent, but active DNA capable of inducing similar specific transformations to type III can now be isolated from the transformed encapsulated cells and their progeny in amounts far greater than that originally required to induce the change. This fact indicates that the specific DNA initially used reduplicates itself in the transformed cells or initiates the reproduction of similar DNA within these cells.

The specificity of DNA is proven by the fact that DNA extracted from type II cells will cause transformation to type II, DNA from type VI cells will induce changes to type VI, etc. For example:

S II→R + (DNA from S VI)→S VI→R + (DNA from S II)→S II.

While these results clearly indicated the existence of specific nucleic acids, each competent to evoke the production of specific polysaccharides, it was also recognized that such specificity must reside only in a small portion of the total DNA fraction. Thus a DNA fraction could also be obtained from unencapsulated R pneumococci by methods identical to those employed in the isolation of active DNA from S III cells, but this DNA from R cells was entirely inactive in capsular transformations. The complexity of the extracted pneumococcal DNA in terms of the number of specific TP's it may contain is further shown by the fact that transformation of other specific characteristics, such as drug-resistance and specificity of somatic protein, can be obtained from the DNA isolated from pneumococci that are identical in their capsular specificity (see below). In what manner such biological specificity of DNA is reflected by chemical differences is virtually unknown. There is some evidence indicating that DNA isolated from different sources may contain different proportions of purine and pyrimidine bases.[10, 11]

Pneumococcal transformations involving intermediate capsular types

In addition to the transformations involving the specificity of the S polysaccharide capsule, transformation reactions have been studied with intermediate capsular mutants of pneumococcus.[12, 13]

[11] Chargaff, E. and Vischer, E., J. Biol. Chem., *177*:405, 1949.
[12] MacLeod, C. M. and Kraus, M. R., J. Exp. Med., *86*:439, 1947.
[13] Taylor, H. E., J. Exp. Med., *89*:399, 1949; Publ. della Stazione Zool. di Napoli, Suppl. to vol. *22*:1, 1950.

These mutants differ from the fully encapsulated S type in the quantity of capsular polysaccharide secreted at the cell surface. Serological and enzymatic studies have shown that qualitatively the polysaccharide of such intermediate mutants does not differ from that of their parent S type. Thus one stable intermediate type designated as S III-1 synthesizes very little type III capsular polysaccharide, another intermediate type, S III-2, consistently secretes a larger amount of type III polysaccharide on the cell-surface, and type S III-N represents the normal, fully encapsulated S type[14]. DNA isolated from each of these three types will transform competent R cells to the condition corresponding to the type from which TP was prepared:

$$\text{R} + (\text{DNA from S III-1}) \rightarrow \text{S III-1}$$
$$\text{R} + (\text{DNA from S III-2}) \rightarrow \text{S III-2}$$
$$\text{R} + (\text{DNA from S III-N}) \rightarrow \text{S III-N}.$$

Furthermore, R cells derived spontaneously from either of the intermediate mutants could be transformed to normal S cells by TP from S III-N. For example:

$$\text{S III-1} \rightarrow \text{R} + (\text{DNA from S III-N}) \rightarrow \text{S III-N}.$$

The foregoing results indicated (1) that a mutation affecting the quantity of polysaccharide produced also affected the TP extractable from the mutant cells, and (2) that each type contained only its particular TP, having lost or altered its original TP during the process of mutation affecting quantitative polysaccharide production. The last point was further verified by the observation that exposure of R cells to a mixture of DNA's isolated from S III-N and S III-1 types yielded both S III-N and S III-1 cells. In addition, the following transformations have been obtained:

[14] Spontaneous reversions from the intermediate capsular types to the fully encapsulated S type may occur, and it is now suspected that previously reported spontaneous reversions from R→S actually represented reversions from an intermediate (I) type with very little capsular polysaccharide, like S III-I, to S (Austrian, personal communication). Type specificity is always retained in such spontaneous reversions. Thus:

$$\text{S II} \rightarrow \text{I} \rightarrow \text{S II}$$
$$\text{S III} \rightarrow \text{I} \rightarrow \text{S III}.$$

S III-1 + (DNA from S III-N)→S III-N

R + (DNA from above S III-N)→S III-N.

In the latter transformation no S III-1 cells were found, indicating that the original S III-1 TP had been replaced by the S III-N TP, or that the former had been modified by the latter.

The demonstration that S III-1 cells can be transformed into S III-N cells also permitted the stepwise transformation from R→S III-N, i.e.:

R + (DNA from S III-1)→S III-1 + (DNA from S III-N)→S II-N,

yielding the same end result as the direct transformation:

R + (DNA from S III-N)→S III-N.

It can be seen that in all these reactions the TP behaved exactly as if it were identical with the gene that controls capsular properties of the donor cells, and transformed cells behaved as if their gene (or genes) controlling capsular properties had been replaced or specifically altered by the TP of the donor cells. However, considerably more complex reactions, indicative of interactions between the TP of the donor cells and the genetic determinants of the transformed cell, were observed in additional studies with intermediate capsular types of pneumococcus.[15] It was observed that two types of encapsulated cells resulted when S III-1 cells were exposed to the TP from S III-2:

S III-1 + (DNA from S III-2)→S III-2 (many)

↘ S III-N (few).

The resulting S III-2 cells correspond phenotypically to the cells from which the TP was obtained, the S III-N cells found represent an entirely different type that does not correspond to the TP employed. Ephrussi-Taylor has designated the latter type of transforming activity as "allogenic," the former as "autogenic." She has furnished several examples of allogenic transformations, all of which suggest that interactions may occur between hypothetical "subunits" of the TP and the factor responsible for polysaccharide production in the "recipient" cell.

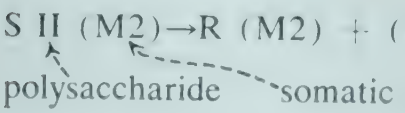
[15] Ephrussi-Taylor, H., Exp. Cell Research, *2*:589, 1951; Cold Spring Harbor Symp. Quant. Biol., *16*:445, 1951.

From the standpoint of infectious diseases it is noteworthy that the above described allogenic transformation can result in the appearance of a highly virulent type (S III-N) from a transformation reaction involving relatively avirulent cells (S III-1 and S III-2).

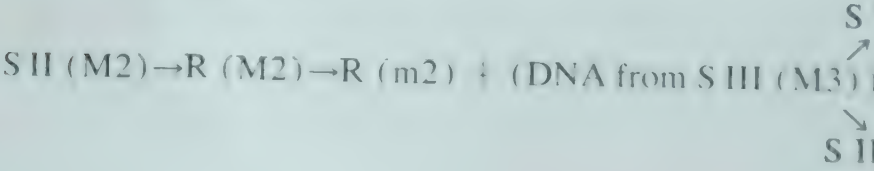
Transformation reactions involving protein specificity, drug resistance and colonial morphology

In addition to transformations involving polysaccharide specificity, it has been possible to demonstrate with pneumococci that other bacterial characteristics can be similarly transformed.

Austrian and MacLeod[1] showed that pneumococci contain a type-specific somatic protein, called M-protein. In the change from S→R this M protein usually remains unaltered. When such R cells were transformed to an S type with heterologous capsular polysaccharide the M protein was found to remain unchanged, e.g.:



This result indicated that the production of type-specific polysaccharide and of somatic M protein varies completely independently, a conclusion which is supported by the fact that pneumococcal strains of the same capsular type but with different M proteins occur naturally. Even though the DNA from an encapsulated type with heterologous somatic protein failed to transform the specific protein in the last cited examples, transformation of M protein became possible when R variants deficient in M proteins were used. Such R types, which we shall call R (m) were obtained following prolonged cultivation of R cells in the presence of anti-M protein serum. When such R (m) cells were exposed to the DNA obtained from an S strain with heterologous capsular polysaccharide and heterologous M protein two types of S clones were recovered, one that produced the M protein of the parent culture and the polysaccharide of the heterologous TP donor, and the other which showed both capsular polysaccharide and M protein identical to those of the TP donor:



The S III (M3) cells represent the products of a double transformation, displaying transformation of both M protein and capsular polysaccharide. It is still questionable whether such a double trans-

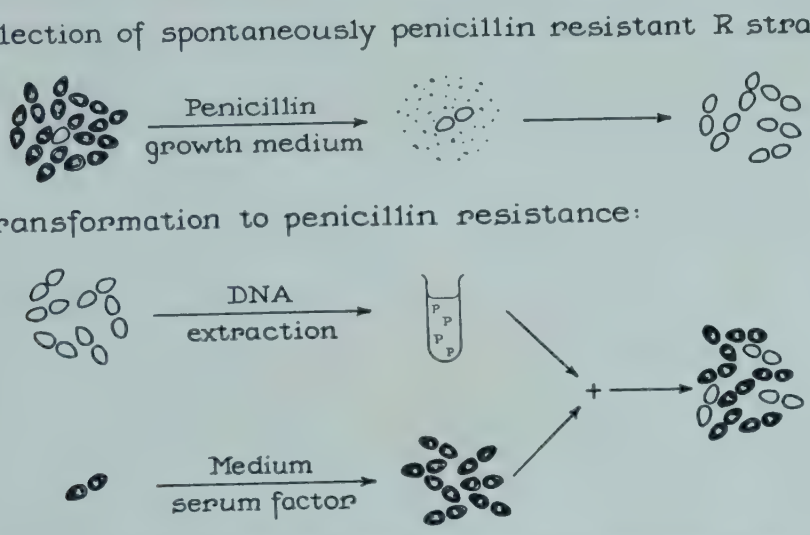


Fig. 61. Transformation to penicillin resistance in *Pneumococcus*. Resistant cells are shown white, sensitive cells black. (R. Hotchkiss.)

formation occurred simultaneously within a single cell or whether it is the result of two successive reactions. But in any event, this double transformation, effected with extracts derived from one

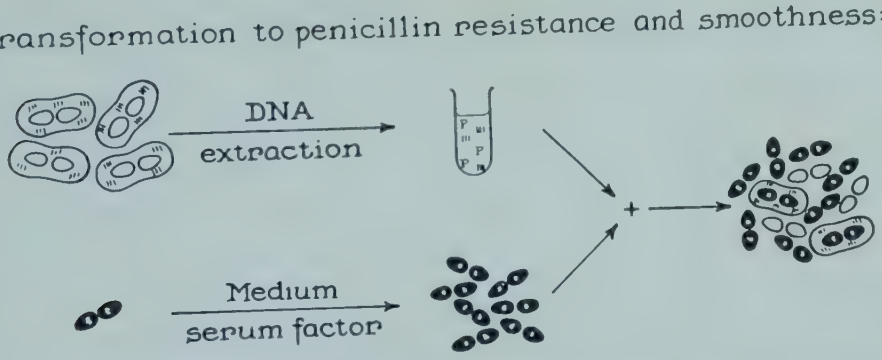


Fig. 62. Transformation to penicillin resistance and smoothness in *Pneumococcus*. (R. Hotchkiss.)

type of S cell, indicates that the active DNA obtainable from each cell must contain a multiplicity of specific transforming principles.

Similar double transformations have been reported in the case of pneumococcal transformations involving transfer of penicillin-resistance to sensitive cells in the presence of DNA obtained from

penicillin-resistant cells[16] (Fig. 61). When penicillin-sensitive (ps) R cells were exposed to the DNA obtained from penicillin-resistant (pr) S cells with heterologous capsular polysaccharide, penicillin-resistant *R* cells and *penicillin-sensitive* S cells with capsular polysaccharide corresponding to the TP donor were obtained (Fig. 62). In addition, however, a very small number of *penicillin-resistant* S cells were found which produced the polysaccharide typical of the TP donor:

$$\text{S II ps} \rightarrow \text{R ps} + (\text{DNA from S III pr}) \begin{cases} \nearrow \text{R pr} \\ \searrow \text{S III ps} \\ \searrow \text{S III pr (double transformation, presumably due to two successive reactions).} \end{cases}$$

The recovery of pr cells in this reaction was 10,000 times greater than that found due to spontaneous mutation under conditions where DNA from pr cells was absent. A remarkable feature of this transformation to penicillin-resistance is the fact that, as in the case of spontaneous mutation to penicillin-resistance, the increase in degree of resistance occurs stepwise (see p. 83). Thus DNA obtained from pr cells 30 times more resistant than the susceptible, exposed cells results in transformation to cells with only 5-fold resistance. Exposure of these latter cells to the same DNA preparation thereupon yields transformed cells showing 12-fold resistance to penicillin, etc. We see here again a parallel between the effects of a succession of TP's and those of a known series of genes.

Colonial morphology is another characteristic of pneumococci that is susceptible to transformation under the influence of DNA isolated from heterologous colonial mutants. We have already referred to several examples of such transformations in discussing reactions involving capsular polysaccharide formation which is closely associated with colonial morphology. In addition, Taylor[13, 17] has studied transformation reactions between so-called extremely rough (ER) and rough (R) variants of pneumococcus. In the absence of R agglutinins, but in the presence of certain serum factors (see p. 208), on exposure of competent ER cells to DNA from R *or* S cells, transformations from ER→R were obtained. Whereas

[16] Hotchkiss, R. D., Cold Spring Harbor Symp. Quant. Biol., *16*:457, 1951; Bull. N. Y. Acad. Med., *28*:346, 1952.

[17] Taylor, H. E., Compt. rend., *228*:1258, 1949.

studies on capsular polysaccharides had failed to reveal any transforming activity in the DNA fraction of R cells, the ER→R transformation demonstrates that both S and R cells contain a TP affecting colonial characteristics. Thus the complexity and multiplicity of specific biological reactions associated with the DNA fraction isolated from any one particular cell type are revealed once more. Since S cells possess TP's for transforming both ER and R cells, it has been possible to transform ER cells to S by exposing them to the DNA from S cells in the presence of R agglutinins. The resulting transformation is a two-step reaction taking place in the same culture:

$$\text{ER} + (\text{DNA from S}) \rightarrow \text{R} + (\text{DNA from S}) \rightarrow \text{S}.$$

Under the conditions utilized in the above experiments spontaneous mutants displaying similar changes in colonial morphology have been detected very rarely and the results must be regarded as representative of true transformation. It is noteworthy that reciprocal transformations have been achieved with R and ER forms:

$$\text{ER} + (\text{DNA from R}) \rightarrow \text{R} + (\text{DNA from ER}) \rightarrow \text{ER}.$$

Taylor has suggested that such reciprocal transformations may involve the exchange between an intracellular TP, which would normally be present in the progeny of the bacterium, and the TP introduced into the transformation culture medium. In this case it might be possible to find transforming activity for ER in filtrates from a culture in which ER cells have been exposed to DNA from R. Such tests have not been reported so far.

Cellular competence and environmental conditions required for transformation

In the course of studies on capsular transformations of pneumococci it was repeatedly demonstrated that only certain strains are capable of being transformed. The factors contributing to this competence are relatively unknown but an involvement of the cells' genetic constitution often has been suspected. Studies on *Hemophilus influenzae* more clearly established such a genetic control of cellular competence in transformation reactions (see below). In addition, the possibility that the structure of the cell surface may affect competence in transformation reactions was indicated by observations on differences in bile solubility between R strains of

Pneumococcus displaying different abilities to participate in these reactions.[18] However, these surface differences may only be a reflection of causally unrelated, associated genetic differences controlling competence. Competence may also be associated with desoxyribonuclease production, because desoxyribonuclease, if normally excreted by the cells or released from autolyzed cells, would inactivate the TP.

In order to obtain transformation reactions with pneumococci relatively complex environmental conditions are required in addition to the use of a competent strain. Reactions *in vitro* require the presence of (1) agglutinating antibody, e.g., R antibodies in the R→S transformation;[19] (2) a dialyzable factor present in serum or serous fluid, replaceable by pyrophosphate, and (3) an additional serum factor which can be supplied by adding the albumin fraction (Fraction V) of bovine serum.[20, 21] Agglutinating antibodies seem to be concerned with the creation of the proper environment, including a reduced O R potential, by causing clumping of the cells subject to transformation. In addition, they probably produce selective effects in the R→S transformations. The role of albumin is still obscure. Equally little is known about the role of the dialyzable serum factor replaceable by pyrophosphate; recent observations indicate that this factor actually may be unnecessary in transformations and only serves to overcome toxic effects that may be present in some systems.[22]

The environmental conditions required for capsular type transformation reactions in *H. influenzae*, *E. coli*, and *Shigella paradysenteriae*, three other species in which similar phenomena have been described, are far less complex. This may be partly due to the higher selective value of the transformed cell types in competition with the original cell type, which would obviate the necessity for a highly selective environment. In *E. coli*, transformation of an R strain to an S type possessing heterologous capsular polysaccharide, under the influence of DNA isolated from heterologous S

[18] For references see Austrian, R., Bact. Rev., *16*:31, 1952.

[19] The presence of agglutinating antibody is not required in the penicillin-resistance transformation.[16]

[20] McCarty, M., Taylor, H. E. and Avery, O. T., Cold Spring Harbor Symp Quant. Biol., *11*:177, 1946.

[21] Hotchkiss, R. D. and Ephrussi-Taylor, H., Fed. Proc., *10*:200, 1951.

[22] Hotchkiss, R. D., personal communication.

cells, has been reported to take place in the absence of any serum factors.[23] Similarly, it has been claimed that type-specific antigenic transformations occurred in *Shigella* in plain nutrient media under the influence of culture filtrates from the heterologous antigenic type.[24] Detailed studies with *H. influenzae* also showed that capsular type transformations (see Table 12) occurred under the influence

TABLE 12. *Influence of transforming principles from each of 6 types of* H. influenzae *on R cells derived from each of these types.**

(From Alexander, H. E., and Leidy, G., J. Exp. Med., *93*:345, 1951)

R CELLS EXPOSED	RESULTS OF EXPOSURE TO TRANSFORMING PRINCIPLES					
	TPa	DNAb	TPc	TPd	TPe	TPf
Ra	Sa	O	O	O	O	O
Rb	Sa	Sb	Sc	Sd	O	O
Rc	O	O	O	O	O	O
Rd	Sa	Sb	Sc	Sd	Se	Sf
Re	O	O	O	O	O	O
Rf	O	O	O	O	O	O

* Letters a through f refer to type from which R cells and transforming principles were derived.

S = type-specific cells; type indicated by small letter.

O = R cells only.

TP = unpurified transforming principle.

DNA = purified transforming principle.

of the specific DNA or TP in ordinary media, and could be initiated even in media that do not support cell multiplication.[25]

The simple requirements for transformation in *H. influenzae* made possible a more accurate analysis of the problem of competence. Alexander and Leidy showed that only a small proportion of the population of R cells is susceptible to the change induced by DNA. The number of these susceptible or competent cells varies among strains and is related to population size. Therefore, it seems that at least one prerequisite of competence involves mutation, i.e., a specific genotype is required to make cells susceptible for transformation.

[23] Boivin, A., Cold Spring Harbor Symp. Quant. Biol., *12*:7, 1947.

[24] Weil, A. J. and Binder, M., Proc. Soc. Exp. Biol. Med., *66*:349, 1947.

[25] Alexander, H. E. and Leidy, G., J. Exp. Med., *93*:345, 1951.

Time required for action of DNA

When competent cells are present in an R population exposed to TP, the transformation reaction occurs very rapidly. In *H. influenzae* the changes that enable an R cell to form a colony of type-specific organisms under the influence of a specific TP are initiated within 3 minutes in an environment that does not permit cell multiplication[25] (see Table 13), i.e., after this period the TP

TABLE 13. *Time required for the action of TPd on Rd cells of* H. influenzae. (From Alexander, H. E., and Leidy, G., J. Exp. Med., *93*:345, 1951)

TIME OF ADDITION OF ENZYME IN RELATION TO INOCULUM	DESOXYRIBONUCLEASE ADDED TRANSFORMATION IN 3 SAMPLES			CONTROLS NO DESOXYRIBONUCLEASE TRANSFORMATION IN 2 SAMPLES		
	Inoculum size			Inoculum size		
	1,000,000	1,000	100	1,000,000	1,000	100
30 min. before	000	000	000	++	++	++
15 " "	000	000	000	++	++	++
5 " "	000	000	000	++	++	++
3 " "	000	000	000	++	++	++
3 " after	+++	000	000	++	++	++
15 " "	+++	+0+	000	++	++	+
30 " "	+++	+0+	000	++	++	++
1 hr. "	+++	0++	000	++	++	++
2 hrs. "	+++	00+	0+0	++	++	++
3 " "	+++	+++	00+	++	++	++
4 " "	+++	++0	000	++	++	++

TPd = transforming principle isolated from type d *H. influenzae*.
Rd = variants lacking type-specific traits selected from type d *H. influenzae*.

becomes protected from the effect of desoxyribonuclease but has not necessarily completed the actual transformation. In *Pneumococcus*, transformation reactions are also achievable following exposure to TP for as little as 15 minutes, but a preliminary four-hour period of growth in the serum medium is necessary to "sensitize" the cells to participate in the transformation reaction.[20] This "sensitization" period does not seem to involve the selection of a mutant, because (1) changes in population size during this period are minimal, and (2) the "sensitization" is lost either by washing the

cells or by additional incubation for two hours. The foregoing reactions were studied by inactivation of the TP through addition of desoxyribonuclease at various intervals following the cells' exposure to TP. On the basis of these results it must be concluded that transformation itself is an inductive and not a selective process. A subsequent stage of selective growth may be needed, however, to permit demonstration of the relatively few transformed cells so induced.

MECHANISM OF BACTERIAL TRANSFORMATION

The actual nature of transformation reactions, which now have been demonstrated to occur in four species, and possibly in others,[18] is still largely a matter of speculation. Evidently, mutation and selection play some role in facilitating the reaction; mutation may predispose the cells toward transformation, and selection enables the transformed cells to establish themselves in competition with the non-transformed parent type cells. The actual process of transformation, however, involves a specific and transmissible alteration of the phenotype of competent cells under the influence of DNA, i.e., a genetic transfer between cells. The simplest explanation is the assumption that the DNA enters competent cells and thereby becomes a permanent part of their genotype, remaining subject, of course, to subsequent mutational changes which are typical for hereditary determinants. This interpretation would be in line with the type of phenomenon uncovered among paramecia, where the killer trait can be introduced into genetically competent cells by "infection" with kappa particles (of DNA nature), which once having invaded the cell continue to propagate therein (see p. 22). The kappa particles, however, propagate within the cytoplasm, whereas in the case of bacterial characteristics subject to transformation it is questionable whether the affected traits are under the control of cytoplasmic factors. A decision on the question whether nuclear or cytoplasmic factors play a major role in transformation will only become possible when such reactions can be studied with bacteria that are also capable of entering into the type of sexual process uncovered in studies with *E. coli,* K-12.

The hypothesis of direct transfer of hereditary determinants into transformed cells, which may be regarded as an "infection" with DNA, is favored by those active in this field of investigation because a DNA with equal biological activity can be isolated subsequently

from the progeny of such "infected" and transformed cells. However, an alternate and more indirect mechanism, whereby the exposure to a specific extracellular DNA may initiate a series of intracellular reactions resulting in the formation or activation of a similar DNA, cannot be ruled out.

Despite the uncertainties about the actual mechanism of transformation *per se*, the analysis of these reactions has made at least three major contributions to bacterial genetics: (1) It demonstrated that directed changes in the hereditary properties of bacteria are possible under specific conditions; (2) it confirmed that desoxyribonucleic acid compounds are genetically active, and (3) it revealed not only that there exist DNA's with different biological specificity but also that a given DNA preparation may affect more than one hereditary property. Genetics will take another step forward as soon as these unique differences in biological specificity can be expressed in chemical terms.

TRANSDUCTION IN *SALMONELLA*

A phenomenon resembling transformation reactions in many respects has been observed in studies with *Salmonella* species.[26] It involves the transfer, or "transduction," of hereditary determinants from one strain to another through a filterable agent which is associated with visible particles of approximately 0.1 microns in size. This phenomenon was detected first in studies designed to test whether recombination may occur between strains of *Salmonella typhimurium*. A total of 20 different auxotrophic mutant strains, obtained by the penicillin screening procedure (p. 94) from different *S. typhimurium* strains, were tested for their ability to yield prototrophs following mixed cultivation. In 9 combinations, out of 79 tested, prototrophs were obtained in large numbers, at a frequency significantly higher than that observed when the parent strains were screened separately. When such strains were maintained physically separated from each other in a U-tube with an ultra-fine fritted glass filter at the base, prototrophs could still be recovered in large numbers from one arm of the U-tube culture. Figure 63 illustrates a typical experiment in which a histidine-requiring strain (2A) and a tryptophan-requiring strain (22A) were employed. If 10^8 cells of either strain were inoculated alone into such a U-tube no prototrophs were recovered, but when 10^8

[26] Zinder, N. D. and Lederberg, J., J. Bact., *64*:679, 1952.

cells of strain 22A were inoculated on one side and 10^8 cells of 2A were inoculated on the other side about 10 prototrophs per million cells were recovered from the side inoculated with 22A cells. No prototrophs were recovered from the side inoculated with 2A cells. It was found that strain 22A carried a phage with weakly lytic activity for strain 2A cells. Under the influence of this phage, which traverses the fritted glass filter, a filterable agent (FA) is released by 2A cells. This FA in turn passes the glass filter and under its influence certain cells of 22A acquire specific heritable properties typical of the FA-donor strain; in the case illustrated this property is the ability to grow without tryptophan. Many other traits have

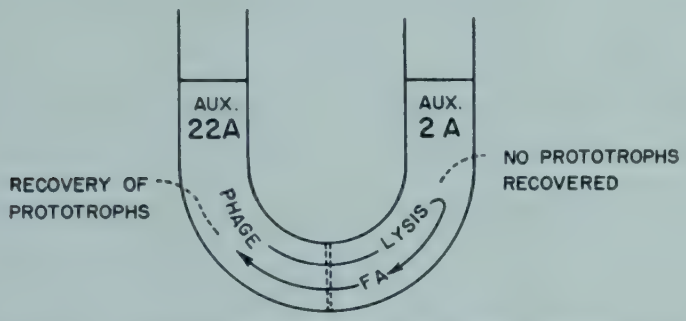


Fig. 63. Diagram of a transduction experiment with *Salmonella typhimurium* employing a U-tube with a fritted glass filter plate in the cross-limb.

been similarly "transduced" with the help of FA, for example, fermentation characteristics, antibiotic resistance, and serologic characteristics (Fig. 64).

A characteristic feature of transduction is the fact that single cells acquire only one of the genetically controlled characteristics of the FA-donor strain. Thus when the FA-donor strain differs from the other strain in two characteristics, e.g., A and B, cells transduced to either A or B may be found in addition to unaltered cells, but simultaneous transduction of single cells to A and B does not occur. This transfer of single hereditary traits from cells of one strain to individual cells of another strain is one of the major differences between transduction and the process of recombination observed in *E. coli;* as we have seen previously, linkage phenomena, involving the recovery of several parent traits in recombinant cells, are typical for recombination.

The filterable agent which is evoked in certain *Salmonella* strains under the influence of phage, and is responsible for the transduction of heritable traits, is associated with particles of approximately

0.1 micron in size which are clearly visible with the help of the electron microscope. The FA is resistant to conditions which are ordinarily believed to sterilize bacterial cultures, i.e., it is resistant to heating at 56 C for 30 minutes and it is not affected by precipitation with alcohol or shaking with chloroform or benzene. In contrast to the inactivation of the TP of *Pneumococcus* or *Hemophilus* by desoxyribonuclease, FA is not affected by exposure to this enzyme. However, this does not necessarily mean that FA is devoid

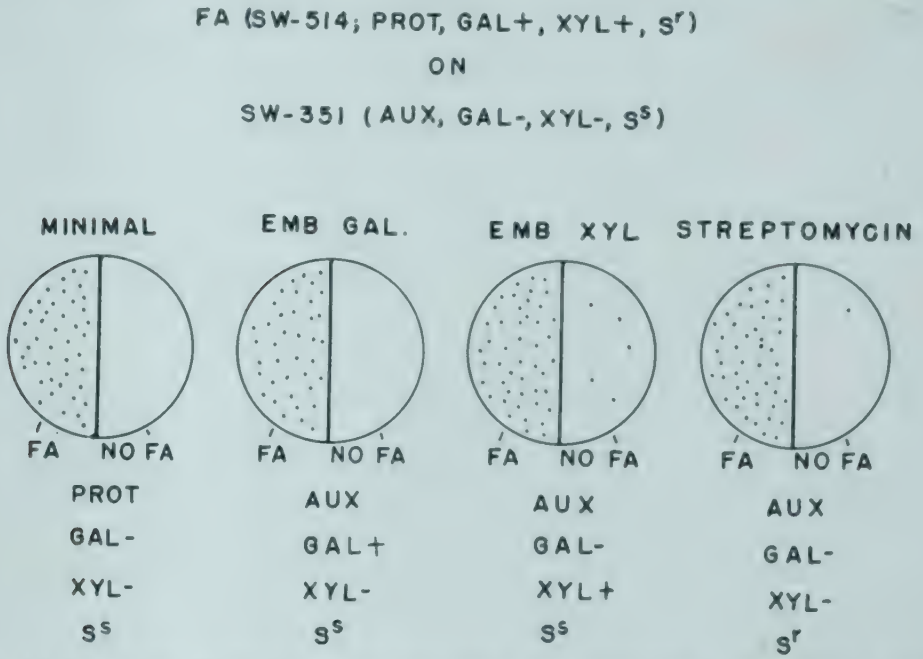


Fig. 64. Diagram illustrating the multiple potentialities of an active filtrate. (From Zinder and Lederberg.[26])

of DNA. It is possible that the DNA, instead of being "naked" as in the case of TP, is protected by some surrounding material.

It has been reported that FA, in addition to being evoked by phage, can also be produced by exposure of the donor strain to lithium chloride, crystal violet, low levels of penicillin or by aging in broth. However, no FA can be obtained from competent strains such as strain 2A, by heat-killing, lysis or autolysis under conditions which are known not to destroy FA activity. Also, filtrates prepared directly from cultures of such strains, without exposure to strains like 22A, are entirely inactive.

Transduction so far has been found to occur only between *Salmonella* strains and species with similar O antigens, specifically between serotypes possessing somatic antigen XII. Thus transduc-

tion between strains of *Salmonella typhimurium* and *Salmonella typhosa,* having similar O antigen but different H antigens, has been possible. This has resulted in what may be considered the first laboratory production of an "interspecific" bacterial hybrid, namely cells displaying the O antigen of *S. typhosa* and H antigen of *S. typhimurium*. This type of cell never has been found to occur naturally.

The filterable particles with which FA is associated were initially believed to be similar to L forms,[27] but later studies[26] have indicated that these particles are identical with bacteriophage. Such phage particles are now regarded as the passive carriers of the transductive material. Since single particles transduce single traits only, it has been assumed that individual particles may act as a carrier for single genes or a portion of a donor's chromosome. However, an alternative mechanism assigning part of the observed specificity to the transducible cell itself, rather than to the FA, cannot be excluded. This would mean that the genotype of the transducible cell may play a role in controlling which new genic determinant can be incorporated into the genotype.

GENERAL IMPORTANCE TO OTHER PROBLEMS

As in the case of recombination, transformation or transduction phenomena can be suspected to have played a role in the evolution of bacteria since they are capable of giving rise to novel combinations of bacterial characteristics. They also may be of significance to epidemiologic problems by yielding types with new combinations of antigenic characteristics and virulence-determining factors when two dissimilar strains coexist within a host under conditions favoring the occurrence of transformation or transduction. Similar genetic transfers between cells, without the requirement for physical contact between the cells involved, have not been observed among higher organisms, with the possible exception of those cases where tumor formation may be caused by viruses. Therefore, transformation reactions are of importance not merely to bacterial genetics, where in addition to mutation and recombination they constitute a supplemental source for new genotypes; the analysis of the mechanisms and the nature of chemical factors involved represent problems of general biological importance.

[27] Lederberg, J., Lederberg, E. M., Zinder, N. D. and Lively, E. R., Cold Spring Harbor Symp. Quant. Biol., *16*:413, 1951.

CHAPTER 13

Bacterial Genetics in Relation to General Bacteriological Problems

THE NEWER DATA ON MECHANISMS RESPONSIBLE FOR BACTERIAL variation must necessarily affect our thinking about a number of general bacteriological problems. Much of this thinking is still speculative, but a science cannot progress unless it occasionally takes stock of the standing of its fundamental problems in the light of progress in a specific field, particularly when that field is as closely related to various other problems as is bacterial genetics. Many bacteriologists (e.g., Hadley, Mellon, Dubos) have examined such problems as pathogenesis, epidemiology, bacterial taxonomy and evolution from the standpoint of variation, and in the preceding chapters we have referred repeatedly to such relationships. We shall examine some of them more comprehensively now.

Metabolism

In Chapter 6 it was shown that studies with spontaneous or induced bacterial mutants displaying changes in synthetic abilities provide an extremely useful tool for the analysis of biosynthetic pathways. Such analyses are of particular value since it has been recognized that similar reactions occur in microorganisms and in higher organisms. Bacterial mutants requiring specific growth-factors also have served to detect new vitamins.[1] We also referred to the initial attempts to analyze with the help of mutants the biochemical nature of factors and steps involved in virulence, antigenicity, and luminescence (p. 99 and p. 127). This type of approach merits further extension, since the biochemical reactions controlling many economically important bacterial characteristics are as yet poorly understood. The nature of these reactions may be clarified by

[1] Davis, B. D., Nature, *166*:1120, 1950.

an analysis of metabolic alterations associated with mutants in which such characteristics have been affected by a one-step mutation. Tests on such mutants should not only include such studies as oxygen uptake in the presence of different substrates and a search for loss of specific synthetic abilities, but should also include tests for alteration of metabolite production. The studies with *Brucella* (see p. 161) illustrate the importance of the latter type of analysis, by showing that antigenically different mutants may not differ in their growth requirements but yet differ quantitatively and qualitatively in metabolite production. Thus some of the unsuccessful efforts to analyze the biochemical basis of differences between related types differing in immunogenicity, virulence, etc. may have been due to an attack at the wrong side of the reaction chain: the difference between such mutants may not lie in their requirements, but what they do with identical growth factors, a difference detectable by an analysis of the end products of metabolism. Once biosynthetic mechanisms involved in immunogenicity have been fully recognized, such recognition may result in the improvement of prophylactic procedures. In the same manner, the understanding of biosynthetic pathways involved in the production of components associated with virulence may make possible new therapeutic procedures involving the blocking of specific metabolic steps. Similarly, metabolic studies on antibiotic-resistant mutants are assisting in the analysis of the mode of action of antibiotics and thereby contribute to advances in chemotherapy. Bacterial genetics also has many actual and potential applications to applied problems in the field of bacterial metabolism. For example, in industrial processes based on bacterial activity, such as fermentations and disposal of waste products, much progress is still possible by applying existing knowledge and methods of isolation and maintenance of mutant strains. In the past, whenever a strain with specific fermentative abilities was looked for, selection of spontaneously occurring mutants was practiced unwittingly by inoculating a given substrate with bacteria. The chances for finding the desired mutant can now be greatly enhanced by the use of mutagenic agents. Similar considerations apply to the isolation of strains that produce antibiotics. The feasibility of obtaining, with the help of mutagenic agents, mutants with higher yields of antibiotic has been amply demonstrated in work with fungi.

Pathogenesis and epidemiology

To what extent are disease processes caused by pathogenic bacteria influenced by genetic phenomena? In Chapter 9, a number of examples of population changes *in vivo* were cited which indicate that following prolonged infection with pathogenic bacteria mutants with altered virulence may establish themselves during the chronic carrier stage. It also has been reported that relatively avirulent mutants tend to establish themselves in naturally resistant and in immunized hosts.[2] More recently it has been possible to demonstrate that population changes involving mutants with altered virulence can occur *in vivo* under the influence of altered amino acid levels, thereby modifying the severity of the infection (see pp. 168, 169). Furthermore, selective effects of specific host tissues favoring the establishment of mutants with different virulence have been noted. For example, *Hemophilus influenzae* isolates from the respiratory tract usually consist of avirulent R types; in contrast, isolates from nerve tissues consist of virulent S types.[3] Such observations suggest that population changes *in vivo*, under the influence of selective host or tissue factors, may play a considerable role in the course of the infective process, as well as in host resistance and susceptibility. However, the extent to which variational phenomena are involved in problems of pathogenesis is still largely a matter of speculation. Yet it is tempting to imagine, especially since metabolites are able to influence the establishment of mutants with altered virulence, that individual or species differences in resistance might be associated with varying ability to maintain conditions suitable for the selection of more or less virulent mutants. Differences in normally existent metabolite levels, in the host's efficiency in removing accumulating metabolites, or in the host's ability to affect metabolite production by the parasite, may all contribute to host susceptibility or resistance by influencing the *in vivo* establishment of bacterial populations of different virulence. Aside from amino acids, or similar metabolites, other specific host factors, including antibodies, may cause the selective establishment of one type of mutant in one host, while in a different host, lacking this factor or possessing

[2] For examples see: Hadley, P., J. Inf. Dis., *60*:129, 1937; Braun, W., Bact. Rev., *11*:75, 1947; also Braun, W. and Hauge, J., J. Immunol., *60*:443, 1948.

[3] Platt, A. E., J. Hyg., *37*:98, 1937; Chandler, C. A., Fothergill, L. D. and Dingle, J. H., J. Bact., *37*:415, 1939.

another selective factor, the establishment of another mutant with entirely different properties may be favored. One host may thus serve as a reservoir for an innocuous bacterial population which, upon transfer to a new host environment, may give rise to a virulent population.

Such population changes may be at the root of many epidemiological problems, even though epidemiologists generally believe that differences in host susceptibility play the major role in epidemic waves. Gowen,[4] for one, has supplied experimental evidence which clearly indicates that genotypic changes of the parasite can play a major role in epidemics quite apart from differences in the genetic constitution of the host. He made 20 single colony isolates from an avirulent culture of *Salmonella gallinarum* and passed 10 of these lines through highly resistant, inbred chickens and 10 through a strain of chickens that is marked by susceptibility to fowl typhoid. During a period of half a year each culture was passed successively through 16 different ten-day-old chicks. Virulence tests were performed throughout the passage experiment. They revealed that sudden changes in virulence occurred in almost all lines during one of the 16 passages. Regardless of the resistance or susceptibility of the host, these changes resulted in a substantial gain in virulence, the total amount of change differing for different lines (Fig. 65). Subsequent tests showed a retention of this change in virulence. Thus, despite significant genetic difference in the natural resistance of the chicks employed, the genetic constitution of the domestic fowl, the natural host for this disease, was sufficient in this case to create the conditions necessary for promoting the selective establishment of *S. gallinarum* mutants with increased virulence.

Epidemiologists are also familiar with the high seasonal incidence of certain diseases. Here again nutritional as well as climatic factors may be responsible for the alteration of selective effects, creating an opportunity for particular mutants to establish themselves under altered environmental conditions (cf. amino acid effects upon virulence of *Salmonella* and *Brucella,* Chapter 9). Similar considerations apply to diseases characterized by quiescent periods without clinical manifestations followed by subsequent relapses. Preceding the carrier stage, avirulent mutants may establish themselves under specific selective conditions existing in iso-

[4] Gowen, J. W., Chapter 19 in *Genetics in the 20th Century,* edited by L. C. Dunn, The Macmillan Co., New York, 1951.

lated foci, such as localized abscesses (see p. 168); subsequent removal from such foci may expose the population to selective

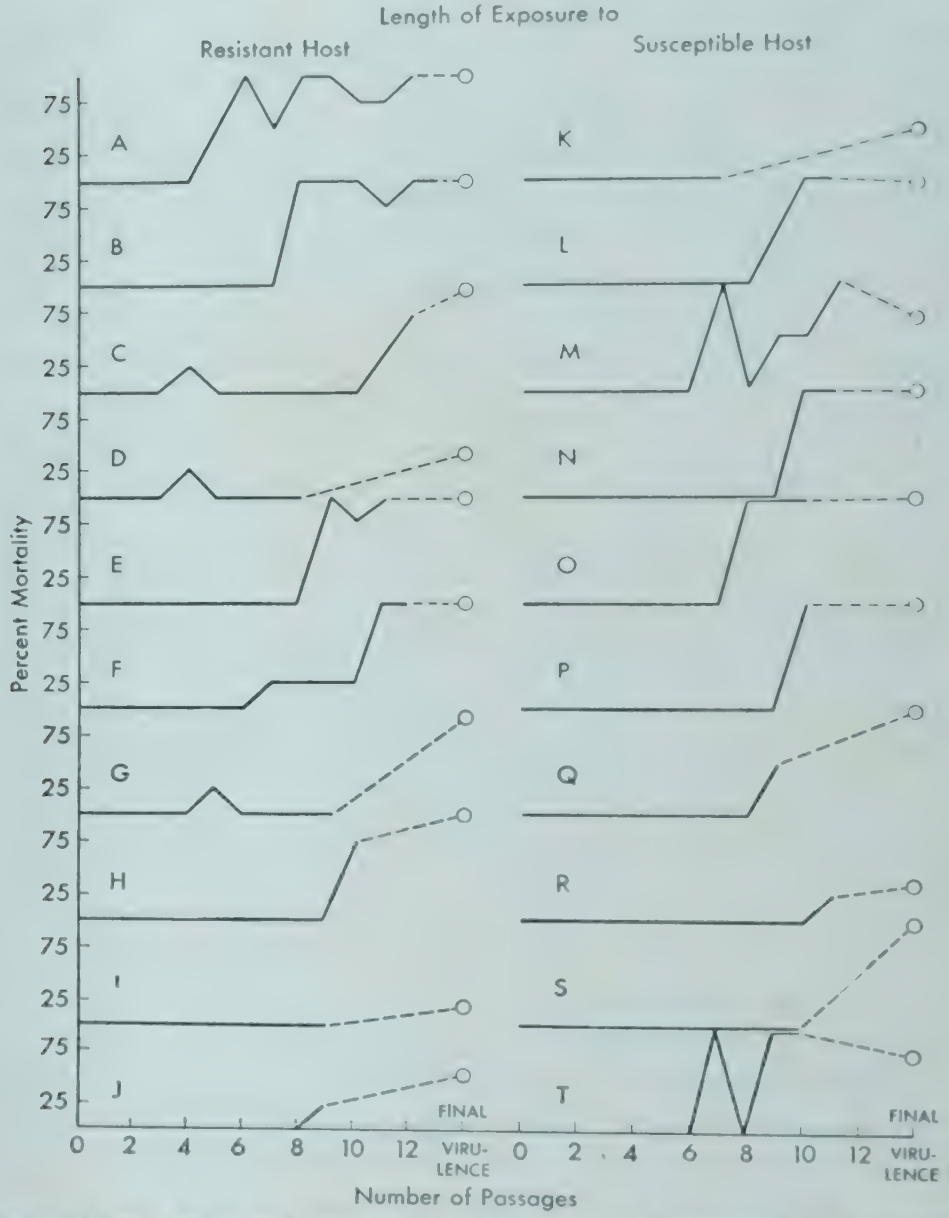


Fig. 65. The effects of repeated passage through susceptible and resistant chickens upon the virulence of an initially relatively avirulent strain of *Salmonella gallinarum*. Note that irrespective of the genetic resistance of the host the virulence of the pathogen increased significantly in all but two of the lines (*I* and *R*). (From Gowen.[4])

effects favoring the establishment of more virulent mutants, thus causing the reappearance of clinical symptoms. Improved recognition of the specific nature of such selective host factors may ulti-

mately permit a novel adjunct to therapeutic measures, namely the artificial creation of an *in vivo* environment favoring the establishment of innocuous, avirulent mutants.

In addition to mutation and selection, a number of other mechanisms uncovered in recent studies can be suspected of contributing to phenomena of pathogenesis and epidemiology. They are:

(1) *Syntrophism.* Studies with *Salmonella* (see p. 131) have shown that simultaneous infection with two dissimilar auxotrophs, capable of "feeding" each other, can result in high virulence, whereas each auxotroph alone grows poorly *in vivo* and shows little virulence. Simultaneous presence of two such strains within one host might account for some natural occurrences of enhanced virulence.

(2) *Growth stimulation of auxotrophs.* Experimental studies with *Salmonella* also have shown that pathogenic variants with specific growth requirements show modified growth and virulence depending on the quantity of the required growth factor present *in vivo* (see p. 131). Seasonal fluctuations in diet, affecting the *in vivo* concentration of a growth factor, accounted for seasonal fluctuations in virulence in the tests with *Salmonella* auxotrophs. It is quite possible that similar effects may occur in nature.

(3) *Recombination.* So far it has not been established whether recombination may occur *in vivo* and in species with known pathogenic variants. However, the possibility remains that recombination between two innocuous strains might result in recombinants with high virulence. Burnet[5] has taken such a mechanism into consideration in discussing possible causes for the influenza pandemic of 1918–19.[6] He states: "The onset of the pandemic . . . was undoubtedly associated with a sharp change in the genetic character of the virus. New type influenza first appeared in an area—Western France—where very large numbers of American troops were mixing for the first time with European service and civilian populations, and there had been considerable influenza of normal type in both continents during the preceding winter. If the experiments on recombination that I described . . . are significant, it seems to me that

[5] Burnet, F. M., Bull. Johns Hopkins Hosp., *88*:157, 1951.

[6] Recombination has been shown to occur among bacterial viruses as well as bacteria. See Luria, S. E. and Dulbecco, R., Genetics, *34*:93, 1949; Hershey, A. D. and Rotman, R., Genetics, *34*:44, 1949.

we must consider quite seriously the likelihood that in this mingling of strains from two parts of the world an opportunity for recombination occurred which provided a form of higher virulence than could have arisen by simple mutation."

(4) *Transformation and transduction.* The occurrence of transformation *in vivo* is well established, yet, as in the case of recombination, its significance in epidemiologic problems is still a matter of speculation. Nevertheless, the demonstration that "allogenic transformation" between cells of moderate virulence can cause the occurrence of cells with high virulence (see Chapter 12) indicates

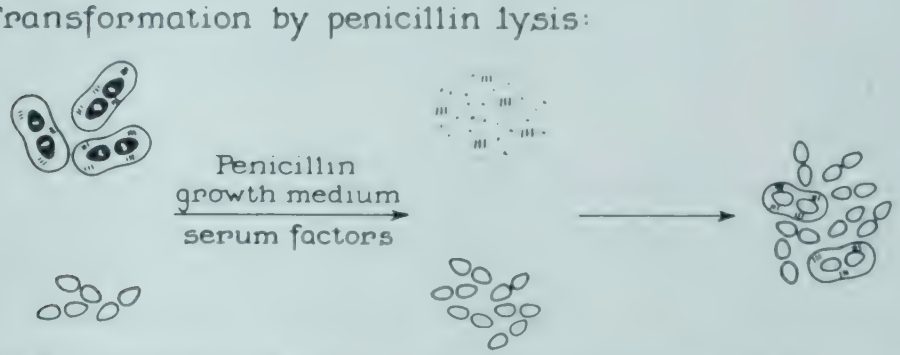


Fig. 66. Transformation of *Pneumococcus* by penicillin lysis. (R. Hotchkiss.)

the potential epidemiologic significance of such phenomena. In addition, Hotchkiss[7] has shown that exposure of sensitive cells to penicillin will yield, by lysis, highly active transforming principles that are capable of effecting a transformation of penicillin-resistant cells which lack characters possessed by the sensitive cells. For example, when unencapsulated, penicillin-resistant pneumococci were grown in the presence of lysates from encapsulated penicillin-sensitive cells, transformation in regard to formation of capsular polysaccharide occurred (Fig. 66). Hotchkiss has suggested that conditions for such transformations by lysates may occur in nature. Thus it is conceivable that in the presence of conditions detrimental to one strain transformation of resistant cells concomitantly present not only might lead to the preservation of traits possessed by the sensitive strain but might also yield cells of altered virulence by superimposing a trait of the sensitive cells upon the genotype of the resistant cells. Similar considerations apply for transduction, especially since the studies with *Salmonella* (see Chapter 12) have

[7] Hotchkiss, R. D., Cold Spring Harbor Symp. Quant. Biol., *16*:457, 1951.

indicated that this phenomenon occurs primarily under conditions unfavorable for the donor strain.

Taxonomy

Currently used schemes of bacterial classification are helpful determinative keys but do not represent natural classification systems based upon the biological relationship between species.[8, 9, 10, 11] Certain easily detected character differences have been chosen to separate "species," "genera" and "tribes," even though such differences may frequently be brought about by a single mutational step,[10] as for example, the difference between the tribes *Escherichieae* and *Proteae* and between the species *Micrococcus (Staphylococcus) aureus* and *Micrococcus (Staphylococcus) albus* in the classification of Bergey's Manual. Similarly, many of the so-called species of Salmonella merely represent mutants from the genetic viewpoint and the same applies to certain *Clostridium* and *Pseudomonas* species, which, though frequently considered as distinctly different from each other, proved to be nothing but relatively stable variants of the same species.[12, 13] The bacterial species thus has little in common with the taxonomic species in higher organisms with recognized sexuality, where species have been defined as "groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups."[14] It is possible that increasing recognition of sexual mechanisms in bacteria may eventually permit a similar approach to more natural taxonomy in this group. In the meantime the present descriptive classification of bacteria can be vastly improved if it is more generally recognized that what is currently described as the "typical" representative of a species merely represents a particular mutant which happens to be predominant under the conditions of laboratory cultivation. However, such types are not necessarily identical to those favored under natural conditions. The lessons learned about the role of selective factors and attendant population changes during primary isolations (see p. 174) must be heeded whenever

[8] Hadley, P., J. Inf. Dis., *65*:267, 1939.
[9] Dubos, R. J., *The Bacterial Cell,* Harvard Univ. Press, Cambridge, 1946.
[10] Luria, S. E., Bact. Rev., *11*:1, 1947.
[11] Braun, W., Bact. Rev., *11*:75, 1947.
[12] Hoogerheide, J. C., J. Bact., *34*:387, 1937.
[13] Gaby, W. L., J. Bact., *51*:217, 1946.
[14] Mayr, E., Adv. in Genetics, *2*:207, 1948.

an attempt is made to isolate a representative biotype whose combination of genetic determinants corresponds to the genotype favored under natural conditions.

Ideally, a description of a bacterial species should include its total potential mutational range. If one compared such mutational spectra of individual species, it would become evident that mutational ranges of species overlap (Fig. 67), resulting in the often confusing observations that the progeny of one species may display characteristics of another species, or even of species currently classified as belonging to different genera. For example, some R mutants of *Pneumococcus* are indistinguishable from certain strains of *Streptococcus viridans*.[15] *Agrobacterium radiobacter (Alcaligenes radiobacter)* and *Agrobacterium tumefaciens (Phytomonas tumefaciens)* lack serologic similarity when their M types are

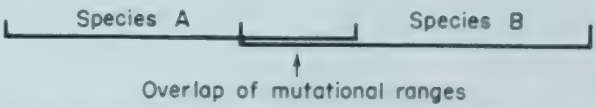


Fig. 67. Diagram illustrating how the mutational ranges of two species may overlap.

tested, but show antigenic identity between their S types.[16] The antigenic gulf usually existing between the non-hemolytic diphtheroids and the hemolytic streptococci has been bridged by variants of the latter, establishing an antigenic continuity between what have been traditionally regarded as distinct genera.[17] Such overlapping of the mutational ranges of diverse species does not mean that there is a continuous genotypic spectrum connecting all bacteria. In other words, the potential genotypic and phenotypic range of each species, i.e., the potential mutations of a given biotype, are normally limited, else we should have been able to observe drastic evolutionary changes in laboratory studies with bacteria. Despite the rapid rate of propagation and the enormous size of attainable populations, changes within initially homogeneous bacterial populations apparently do not progress beyond certain boundaries under experimental conditions. Such boundaries are presumably controlled by the lack of selective value of certain mutants and by the occurrence of lethal mutations, i.e., an arbitrary mutational step

[15] Paul, J. R., J. Bact., *28*:69, 1934.
[16] Coleman, M. and Reid, J. J., J. Bact., *49*:187, 1945.
[17] Mellon, R., J. Immunology, *60*:91, 1948.

superimposed upon the existing genotype will fail to yield viable progeny. However, in nature, countless generations of bacteria have been subjected to a great variety of environmental conditions throughout the ages, which greatly increased the opportunity for the rare occurrence of a particular genotype (chance association of certain genes) that would yield viable progeny with selective value when an additional mutation occurred. Such rare mutants, overstepping the normal boundaries of a species' mutational range, then could attain evolutionary significance.

Evolution

As the foregoing statements indicate, mutation and selection can account for evolutionary changes in bacterial populations. Our present concepts of evolution among bacteria thus reflect Darwin's theory of natural selection, which pioneered the way for explaining adaptive evolutionary changes among higher organisms. But, just as the causal analysis of evolutionary changes in higher organisms failed to make its greatest progress until the role and behavior of spontaneous mutation were recognized,[18] evolutionary theories for bacteria lacked a causal basis until the occurrence of mutations was demonstrated. It can now be stated with some assurance that in bacteria, as in higher organisms, mutations furnish the major source of novel genotypes on which natural and artificial selection can act. In addition, recombination, transformation and transduction, provided they occur at sufficient frequency among bacteria, may expedite the occurrence of particular genic combinations with unique selective values and potential evolutionary significance. In preceding chapters we have discussed many examples that illustrate how genetic heterogeneity, usually existing in bacterial populations because of the constant occurrence of spontaneous and undirected mutations, plus the selective effects of the environment can result in the rapid establishment of mutants especially adapted to the existing environmental conditions.

It is evident that in the course of evolution bacteria have been faced repeatedly with novel environments, new hosts, new substrates, etc. A mutant capable of establishing itself in such a new environment may always have been within the potential mutational range of the parent type, but as long as the appropriate environ-

[18] Dobzhansky, Th., *Genetics and the Origin of Species*, 3rd ed., Columbia Univ. Press, New York, 1951.

mental conditions for endowing it with high selective value were lacking, it would have had as little evolutionary significance as a lethal mutation. Yet once it has become established in the new and appropriate environment, it can serve as a source of additional mutations which, superimposed upon a previously non-selected genotype, may give rise to mutants that are sufficiently different from the original stem-type to be regarded as novel species. Even now we are witnessing in nature the establishment of novel mutants which had no survival value 10 or 20 years ago. We are referring to the antibiotic-resistant mutants, which, although within the potential mutational range of many species, had no survival value prior to the days of therapy with antibiotics. A streptomycin-dependent mutant may be regarded as the most extreme example in this category.

Several microbiologists have speculated on the direction of evolutionary trends, especially in regard to nutritional requirements. The frequent occurrence of auxotrophic mutants with increased growth requirements illustrates how evolutionary changes may have proceeded from autotrophic organisms with high synthetic abilities towards organisms with increased nutritional requirements. Knight[19] and Lwoff[20] have argued in favor of such increasing specialization. Many indications of such a regressive physiological evolution can be observed among bacteria; for example, the transition from coliform to typhoid to dysentery bacilli, appears to involve successive losses of fermentative capacities and synthetic abilities.[10] Similarly, it has been suggested that evolution in the *Salmonella* group has involved successive, spontaneous losses of antigenic components.[21, 22] Also, anaerobes may have evolved from aerobes by mutations affecting enzyme systems such as cytochrome oxidase. It is easy to understand how increasing parasitism enhanced the selective value of those mutants that were unable to synthesize a required growth-factor which now was made available by the host. Since mutation rates from prototrophy to auxotrophy are usually higher than reverse mutations reestablishing synthetic abilities (see p. 63) mutation pressure and selection will inevitably lead to a

[19] Knight, B. C. J. G., Med. Res. Council (Brit.), Special Rept. Series, 210, 1936.

[20] Lwoff, A., *L'évolution physiologique*, Masson, Paris, 1943.

[21] White, P. B., Med. Res. Council (Brit.), Special Rept. Series, 103, 1926.

[22] Bruner, D. W., J. Bact., *64*:138, 1952.

disappearance of the original prototrophic organisms. Furthermore, if the environment is capable of supplying the required end product of a reaction chain, e.g., thiamine, mutations producing blocks of other steps in the same reaction chain, e.g., synthesis of thiazole and pyrimidine, can accumulate without adverse selection and thus cause the successive loss of the entire series of reactions involved in the synthesis of the end product.[10, 23] Thus if the reaction chain for the synthesis of a growth-factor D is A→B→C→D, each step being controlled by a specific gene, a mutant A→B→C↮D can establish itself in an environment supplying D. Unless the synthesis of C serves some independent need not satisfied by the availability of D, a subsequent mutation blocking B→C will also have a selective value. Finally, a mutant unable to synthesize the intermediate B from A, which can be written A↮B↮C↮D, will have a chance to establish itself in an environment supplying D. In order to reverse this process, permitting organisms to synthesize D in an environment free of D, three simultaneous mutations, reestablishing reactions A→B, B→C, and C→D, would be required. The probability for the occurrence of such an event is exceedingly small. Even if true reverse mutations, e.g., A→a→A, do occur, and we have stressed previously that their occurrence may be rare (p. 96), it can be expected that selectively advantageous mutations at various other genetic loci may occur prior to the time that a reversion occurs restoring the original gene activity and phenotype A. Therefore, despite the restoration of the original phenotype it is unlikely that the original genotype would recur. As Luria[10] has phrased it: "We must keep in mind that natural selection is always at work on the genotype as a whole rather than on individual characters, with the result of making a strain better fitted to life in the environment in which it has grown for any length of time."

One can now ask how the complex reaction chains of synthetic processes in bacteria evolved in the first place, if the trend towards specialization, including increasing nutritional requirements, is so difficult to reverse. Horowitz[24] has attempted to furnish an answer to this question. Starting with the assumption made by Oparin[25] and others that complex organic systems developed prior to the advent of the first self-duplicating units of life, Horowitz proposes

[23] Beadle, G. W., Physiol. Rev., *25*:643, 1945.
[24] Horowitz, N. H., Proc. Nat. Acad. Sci., *31*:153, 1945.
[25] Oparin, A. I., *The Origin of Life*, The Macmillan Co., New York, 1938.

that the first living entity was heterotrophic, reproducing at the expense of the organic molecules present in its environment. The evolution of synthetic reaction chains, making the organisms less dependent upon pre-formed organic molecules, may then have taken place backward from the final product. Let us assume again that product D is required by the initial organisms, which are unable to synthesize it but can obtain it from the environment. In addition, the environment would contain compounds C and B, capable of yielding D in the presence of the appropriate enzymatic catalyst. As the environment becomes depleted of D, because of its utilization by our hypothetical stem-type, any mutant that can carry out the reaction B + C→D will attain a high selective value and can establish itself. Subsequently the depletion of the intermediate compound B would promote the selective establishment of a mutant capable of synthesizing B from other available compounds, e.g., A + A′→B. Thus the evolution of a reaction chain A + A′→B→C→D could be initiated. Owing to the lack of B in the environment a reverse mutation, reestablishing the requirement for B, would be a lethal mutation. In this manner autotrophic organisms could have evolved from heterotrophs, and Horowitz suggests that similar evolutionary changes might still be occurring, since complex organic molecules are available in the present-day environment.

Thus the evolution of bacteria may have progressed, and probably continues to progress, in either direction: towards autotrophy or towards heterotrophy.

From the above considerations it becomes evident that complex evolutionary changes can be attributed to undirected changes of nuclear determinants. To what extent changes in extra-nuclear factors may have contributed to evolution is still unknown. Yet the possible role of cytoplasmic factors cannot be excluded, as illustrated by the findings with *Paramecium* which demonstrate that the incorporation of self-duplicating, virus-like particles into genetically competent cells can cause the manifestation of new heritable properties (see p. 22). Particularly in view of the fact that the symbiotic association of bacteria with phage (lysogenicity) is a not infrequent phenomenon,[26] the possibility of the occurrence of similar mechanisms in bacteria cannot be overlooked. The answer to this problem remains one of the future tasks of research in bacterial genetics.

[26] See Lederberg, J., Chapter 3 in *Bacterial Physiology*, ed. by Werkman, C. H. and Wilson, P. W., Academic Press, Inc., New York, 1951.

Index

Extended discussions are indicated by **bold-face** figures

A

Abscesses, 167–168, 220
Achromobacter fischeri, 194
Acquired characteristics, **3,** 30, 32, 60–61
Acriflavine, 25, 69, 118–120
Adaptation, 28, 96, 138, 225
  direct, 32, **33–35,** 50, 53, 58, **60–61**
  simultaneous, 180–181
Adaptive
  enzyme formation, 26–28, 101–102, 178–183
  growth, 163
Adenosine, 80
Aerobacter aerogenes, 186
Aerobes, 226
Agar,
  complete, 91
  EMB, 102
  MacConkey's, 106, 110–112, 123
  minimal, 91
  2–1, 106
Agglutinability, 133–135
Agrobacterium radiobacter, 224
  A. tumefaciens, 154, 224
Alanine, 120, 124, 133, 148, 152–159, 161, 167–168
Albumin, 208
Alleles, **9–10,** 12–14, 121, 136, 195
  multiple, 195
Amino acids, 58, 99, 133, 152, 168
  effect upon population changes *in vivo*, 168–170, 218
  requirement for, 95, 128, 130–131, 187
Anaerobes, 226
Anaphase, 5–6
Anthranilic acid, 97, 99
Antibiotic production, 217
Antibiotic resistance, 54, 61, 63, **81–88,** 205–206, 213, 217, 226
  and colonial morphology, 110–111, 133
  cross resistance in, 86–87
  determination of spectra of, 55
  in relation to therapy, 87–88
  isolation of mutants with, 81, 83–85
  non-mutational increase in, 87, 177
  one-step increase of, 81
  stepwise increase of, 83
Antibodies, 47, 158, 165, 208, 218
Antigen,
  flagellar (H), 63, **114–117,** 215
  group, 63, **114,** 116–117, 140–141
  somatic (O), **114–116,** 132, 199, 204, 214–215
  specific, 63, **114,** 116–117, 140–141
  Vi, 131–132
Antigenic characteristics, 25, 104, **114–121,** 127, 134, 213, 215–216, 224, 226
  and virulence, 123, 134
  cause of changes in, 115–117, 127
  changes of, 25, 96, 101, **114–121,** 133–137, 150, 154–155
  development of, 99
  mutational origin of changes in, 60, 114, 124
  of auxotrophic mutants, 127–133
  rate of mutations affecting, 117, 124–126
  recognition of, 106, 117–118
  simple indicators of, 118–120

Antigenic characteristics, transformation in *Paramecium*, 23–25, 127
types of, 114–115, 198
Anti-mutagens, 80
Antiserum, 23, 60, 117, 127, 158, 175, 199
Apoenzyme, 27
Arabinose fermentation, 187
Asexual reproduction, 6, 8, 21, 32, **46**
Associated changes, 104, 107, 109, 118, **123–124, 133–137**
in antigenic complexes, 116–117
Aureomycin resistance, 82, 86, 88
Autocatalytic processes, 6, 20, 22, 24–27, 198, 227
Autogamy, 21, 42
Autotroph, 226, 228
Auxanographic method, 95
Auxotrophic mutants, **90–100,** 127–132, 143, 186, 194, 212, 226–227
antigenicity and virulence of, 127–132
applications of, 99–100
assaying requirements of, 94–95
colonial morphology of, 110
growth stimulation of, 131–132, 221
isolation of, 91–94
Azide resistance, 191
Azotobacter, 99

B

B type, 42, 88–90, 194
B/r type, 41, 88–90
Bacillus anthracis, 109, 134–135
B. cereus, 45, 183
B. megatherium, 43, 45–46, 134, 146
B. subtilis, 181
Bacterial variation,
cytological basis of, 38–48
history, 32–37
proposed mechanisms of, 33–37
Bacteriological technique, 170–175
Bacterium stewartii, 103, 126, 186
B. tularense, 106–107, 118–119, 123, 153, 158, 172
B. typhosum, 58, 63, 128, 131–132, 134
BAL, 80
Bergey's Manual, 223
Biochemical mutants, **90–103,** 226–228
Biologic assays, 99
Biosynthesis, 4, 18–19, **96–99,** 109, 113, 115–117, 127, 131, 133, 135, 180–181, 217, 227
Biosynthetic steps, sequence of, **99, 180–181,** 216, 227–228
Biotype, 224
Blocking of biosynthetic steps, **97–98,** 115, 128, 132, 136–137, 227–228
Brucella, 106, 113, 118, 124, 133, 151–162, 167–168, 217
B. abortus, 120, 124–125, 134, 148, 150–155, 159–160
B. melitensis, 159
B. suis, 155–156, 166

C

Caffeine, 80
Camphor, 9, 46
Capsule, 108, 115–116, 122, 134–135, 198–206
Carbohydrate, 102, 116, 134, 146
Carcinogens, 69
Carriers, 167, 218–219
"Carry-over" effect, **162,** 173, 176
Caryophanon latum, 45
Cell, 38–48
arrangement in colonies, **104–105,** 113
count, 148
fusion, 188–189, 192
morphology, 104, 133–134
surface, 104, 135, 202, 207
wall, 6, 47–48
Cell division,
and mutation rate, 67
modes of, 47–48
Centriole, **6,** 43–44
Centromere, 5
Chelating agents, 157
Chemostat, **67,** 139, 143, 165
Chicken, 166, 194, 219–220
Chloramphenicol resistance, 83
Chromatids, **7,** 184–185
Chromatinic elements, 40, 44
Chromatography, 152
Chromosomes, **4–9,** 11–12, 14–15, 30, **43–46,** 184–186, 189–190, 195, 215
distribution of, 5–8
duplication of, 5–9
homologous, **5,** 7–9
numbers of, 5–9, 45, 193
of salivary glands, 4, 186

Chromosomes, structural changes of, 14–15
Citrate, 101, 164
Clone, 21, 51, 53–54, **63,** 64, 123, 138, 145–146, 159, 172
Clostridium, 134, 223
Colchicine, 9
Collateral sensitivity, 86–87
Colonial morphology, **104–114,** 124–127, 133–136, 145, 147, 150, 153
as indicator of antigenic characteristics, 118, 120, 173
as indicator of changes in resistance and growth requirements, 109–112
as indicator of population changes, 114, **150,** 172
as indicator of virulence, 123
detection of mutants affecting, 104–107
environmental effects upon, 112–114, 177–178
rate of mutations affecting, 63, 124–126
significance of changes in, 108–109
transformation of, 206–207
types of, 106–109
Colonies, 48, 76, **104–105,** 145–148
inspection of, 105–106
isolation of single, 171–172
secondary, 111–112, **146**
sectored, 76–77, 146–147
Competence
in enzymatic adaptation, 179
in recombination, 193
in transformation, 207–209
Competition
among cytoplasmic reactions, 24, 181
between enzyme-forming systems, 27, 181–182
between mutants, 111, 138, 142, 181
Conjugation, 21–22
Continuous culture, 67, 139
Corynebacterium diphtheriae, 134, 154
Cosmic rays, 67
Count,
total cell, **148–149,** 174
viable cell, **148–149,** 151–152, 174
Cross resistance, 85–87
Crossing-over, 12, **184–186**
frequency of, 185
Culture filtrate, 151–152, 154, 192, 197, 209, 214
Culture system,
closed, 151, 153–154, 165
open, 167
Cyclogenic theory, 35–36
Cysteine, 80
Cytochrome oxidase, 25, 87, 226
Cytogenetics, 48
Cytological observations, **5–9,** 30, **38–48,** 76, 184, 186
Cytoplasmic factors, **20–27,** 75, 87, 101, 103, **126–127,** 181, 195, 211, 228
Cytoplasmic inheritance, **20–26,** 30

D

Darwin's principles, 28, 225
Dauermodifikationen, 33–34
Deletions, 14–15
Desoxycholate, 125
Desoxyribonuclease, 208, 210, 214
Desoxyribose nucleic acid (DNA), 4, 23, 39–40, 197–212, 214
Diagnosis, 114
Diauxie, 181–182
Diphtheroid bacteria, 40, 108, 224
Diploid, **5–8,** 12, 14, 46, 193, 195
Disease, 121, 132, 204, **218–223**
Dissociation, **35–36, 109,** 150–151
index, 151
Division
of cells, 5–7, 13, 41, **46–48**
of nucleus, 4–8, 40–44
DNA. See DESOXYRIBOSE NUCLEIC ACID.
Dominance, **9–11,** 14, 187, 195–196
Dosage effects, 70–73
Drosophila, 1, 5, 20–21, 185
Duplications, 14–15
Dwarf type, 108, 154

E

Eggs, 166, 193
Enteric bacteria, 106, 112, 125
Environmental conditions,
changing, 144–164, 225
constant, 139–143
necessary for transformation, 208–209
Environmental effects, **2–4,** 12, 15, 30
on colonial morphology, **112–114,** 177–178
on cytoplasm, 24

Environmental effects, on cytoplasmic factors, 23–25
on growth, 144
on pigmentation, 103
on population changes, **150–158,** 161, **176–183,** 218–220
on recombination, 193–194
on selective value, 28, 31, 225–226
Enzymatic adaptation, **26–28,** 101–102, **178–183**
kinetics of, 181–183
vs. mutational changes, 101, 179
Enzymatic processes, 58, 61, 101–102, 115, 178–183, 226, 228
and substrate, 26, 101–102, 178
control by genes, 19, 27, **179–180**
inactive, 19
Enzymes,
adaptive, 101, 179
constitutive, 101, 179
Epidemics, 196, 215, **219–222**
Equilibrium
after use of mixed inocula, 159–162
between mutants of equal growth rate, 139–141
in periodic selection, 142–143
in phase variation, 115, 140
Escherichia coli, 41–42, 45–46, 49–50, 52–54, 63, 67, 70–74, 76–78, 80, 85–91, 93, 98–102, 105, 110–111, 113, 116, 118, 134, 136, 142, 147, 164, 178–180, 182, 186–195, 208, 223
Evolution, 20, **28–29,** 31, 196, 215, **224–228**
Extra-chromosomal factors, 19–20, 228

F

Fermentation, 26, 54–55, **100–103,** 134, 145–146, 213, 217, 226
Fertilization, 12
Feulgen reaction, 39
Filamentous cells, 42, 90, 105
Filterable agent (FA), 212–215
Filterable forms, 47
Flagellae, 3
Fluctuation test, 50, **52–54,** 62–65, 73, 118, 124–125, 179
Fungi, 8, 13, 18, 28, 171

G

G type, 108
Gaffkya tetragena, 134
Galactose fermentation, 26, 102, 187
Galactoside, 178
Galactozymase, 26, 102
Gametes, **6–8,** 10–11, 20, 184–185, 193
Generation time, 67, 142
Genes, **4,** 12, 15, 30, 184, 195, 203, 206, 215
action of, **15–20,** 25–26, 30, **96–97**
affecting quantitative characteristics, 16, 195
and enzymes, 19
changes of, **12,** 14–15, 18, 62, 121, 126
duplication of, 6
linked, 11, **184–186**
mutant, 12–13
potency of, 82–83
response to mutagen, 73, 77
site of, 4, 190
transmission of, 9, 12
Genetic principles, 1–31
Genotype, **2–3,** 5, 16, 24, 28, 138, 161, 176, 184, 186, 193, 209, 215, 224–227
Genus, 223–224
Glucose fermentation, 102, 180
Glucozymase, 102
Gradient plates, 83–85
Growth,
adaptive, 163
cessation of, 145, 151, 174
continuous, 139–143
cycle of, **148–149,** 181
logarithmic, 65, 117, 139, 141, 148, 157, 170
residual, **65–66,** 144, 194
selective, 144
Growth rate, 28, 41, 111, 117, 139–143, 145, 149–150
of mutants, 62, 64, 111, 117, **139–145,** 148–150
Growth requirements, 18, 28, 54, 69, 77, **90–100,** 108, 131, 217, 226–228
assaying of, **94–95,** 174
Guanosine, 80
Guinea pig, 166–168

H

Haploid, **7–8,** 12, 42, 45, 189, 195
Hemophilus influenzae, 207–210, 214, 218
H. pertussis, 63, 134

Hereditary characteristics, 29
controlled by cytoplasmic factors, 23, 25
nature of, 2–4
stability of, 12, 67
transmission of, 5, 11
Hereditary determinants, **2–6,** 9, 197
site of, 4–5
stability of, **12, 49,** 62, 67, 77
transmission of, 2, 5, 9
Heterogeneity in populations, 28, 111–112, 118, **138,** 169–170, 171–172, 174, 225
Heterokaryosis, 13–14
Heterotroph, 228
Heterozygous, 193, 195
Histidine requirement, 63, 111, 128, 131, 142–143, 163, 212
History of bacterial genetics, 32–37
Homogeneous cultures, 64, 111, **138,** 170–171, 174, 224
Host, 121, 131, 144, 165–168, 218–220, 226
defense mechanisms of, 144, 166
factors, 165, **167–170,** 174, **218–220**
Hybrid, 215

I

I type. See INTERMEDIATE TYPE.
Immunogenicity, 112, 118, 128–130, 172, 217
Independent changes, 118, **133–137**
Indole, 97, 99
Infection, **166–170,** 211, 218
effects of diet on, 170
Influenza, 221
Inhibitory effects, 144
of metabolites, 148, **152–154**
of parents on mutants, 65, **163–164**
Inoculum, 139, 148, 160, **172–173**
mixed, 142, **159–162**
Inosine, 80
Interactions between
different biosynthetic pathways, **98,** 117
gene-controlled processes, 17
genes, **15,** 116
genes and cytoplasmic factors, 23, 26
hereditary determinants and environment, 2
mutants in mixed populations, 159–162
Interactions between transforming principles, 203
Interactions, biosynthetic, 4
in synthesis of enzymes, 181–182
Intermediate type, **108,** 115, 122, 166, 202–204
Invasiveness, 121
Inversions, 14–15
Isoleucine, 98, 136
Isonicotinic acid hydrazide resistance, 82–83

K

K-12 strain of *E. coli*, 186–194
Kappa, **22–23,** 211
Ketovaline, 98
Killers, **21–23,** 211
Klebsiella pneumoniae, 128, 132, 134

L

L-forms, 36, **46–47,** 108, 215
Lactobacillus, 99, 113
Lactose fermentation, 76, **100,** 102, 146–147, 180, 187, 195
Lag, 60–61, 144, 148
in enzymatic adaptation, **101,** 181
mutational, 77–79
phenomic, 13, 64–65, **73–75,** 77, 79, 196
physiologic, 75
Lamarckism, 32
Lederberg's indirect selection test, 55–57
Lethal effects, 73, 79
Life cycles, **33–36,** 46, 108–109, 133, 154
Light, 69, 79
Linkage, 11, **184,** 188–190, 195
maps, 185
Loci, **9,** 24, 129, 195, 227
Luminous bacteria, 99, 216
Lyophilization, 171
Lysogenicity, 192, 228

M

M concentration, 151
Maltose, 26, 101, 180, 187
Maltozymase, 26
Manganous chloride, 69
Mannitol fermentation, 187
Markers, 99, 103, 187, 194, 199
selected, 190–191

Markers, unselected, 190
Matt type, 108
Maturation division. See MEIOSIS.
Medium,
  complete, 91, 113
  for inspection of colonial morphology, 106
  indicator, 100, 102
  liquid, 145, 148–164, 170, 175
  minimal, 91, 113
  solid, 145–148, 170, 175
Meiosis, **6–8,** 12, 46, 184, 188
Mendelian principles, 10–12
Metabolic studies, 173–174, 216–217
Metabolites,
  accumulation of, 144, **153,** 157–158, 174, 217
  affecting population changes, 146, **151–158,** 174, 218
  assaying of, 99, 152
  growth dependent on, 146
  inhibitory, 146
  intracellular levels of, 24
  synthesis of, 19, 157
Metaphase, **5–6,** 43–44
Mice, 128–129, 131, 199
Micrococcus albus, 45, 223
  M. aureus (*pyogenes*), 58, 223
  M. cryophilus, 44–45
Microcolonies, 89–90, 124
Mitochondria, 25
Mitosis, **5–6,** 8, 43–45
Modifications, **2–3,** 30, 103, 112–116, 162, **176–178**
Modifier genes, **17,** 116–117
Monomorphism, 35
Mucoid type, 36, **108–109,** 113, 119–120, 157, 166, 224
Multinucleate cells, **13–14,** 41–42, 75–76, 146, 196
Multiple factor hypothesis, 16
Multiple irradiation method, 89
Mutagenesis, 67, 80
Mutagens, 14, 49, **69–73,** 77, 79–80, 164, 171, 217
  indirect effects of, 78–80
Mutant frequency, 66
Mutants, 49
  antigenic, 104, **114–121**
  apparent, 94
  auxotrophic, 90–100
  biochemical, 18, **90–103**
Mutants, colonial morphology, 104–114
  delayed, 79
  detection of biochemical, 55
  fermentation, 100–103
  in evolution, 28, 225–228
  isolation of, 55, 79, 81, 83, 88–89, 91–94
  pigmentation, 103
  representative types of, 81–137
  resistant to inhibitory agents, **81–90,** 144, 217
  spontaneous vs. induced, 69
  virulence, 121–124
  zero-point, 79
Mutation, **12,** 30, **49–68**
  affecting different levels of gene-controlled processes, 17–18, 135–136
  and selection, 33–34, **36,** 96, 126–127, **138–175,** 211, 221, 225
  delayed, 77–79
  differentiation between modification and, 112–114, 179
  effects of reducing substances on, 79–80
  evidence for occurrence of, 50–60
  in multinucleate cells, 75–77
  induced, 14, 29, **69–75**
  influence on rate of another, 137
  lethal, **17,** 29, 224, 226, 228
  of plastids, 21
  origin of, 12, **67–68**
  pattern, 143
  phenotypic delay of, 73–75
  pressure, **139,** 142, 144, 160, 226
  reverse, 17, 23, 77, **95–97,** 115, 117, **120–121,** 139, 164, 187, 202, 226–228
  spontaneous, 14, 29, **49,** 62, **69,** 127, 225
Mutation rate, 12, 14, 49, **62–67,** 69, 82, 101, 103, 115, 124–126, 139–142, 150, 169
  and cell division, 67
  and lethal effects, **73,** 79
  determination of, **62–67,** 73, 117
  vs. mutant frequency, 66–67
Mutational lag, 77–78
Mutational range, 224–225
Mycobacterium tuberculosis, 113, 134, 153,166

N

Natural occurrence, 108–109, 138–139, 223
Neomycin resistance, 83
Neurospora, 18, 20, 97–99, 136
Newcombe's spreading experiment, 50–52
Nitrogen fixation, 99
Nitrogen mustard, 14, 69, 129
  resistance to, 90
Noguchia granulosis, 134
Nuclear components, **4,** 9, 29, **44–46**
Nuclei, 4, 38–43
  division of, **4–6,** 13, 41–42
  fusion of, 42, 46
  membrane of 6
  number of, 13, **41–42,** 75–77
  staining of, 39
  transfer of gene products from, 19
Nucleoproteins, 4
Nucleosides, 95
Nucleotides, 95
Nutrients, exhaustion of, 144–145, 148

O

Oblique lighting, **106,** 108 110, 123, 172
Offspring. See PROGENY.
One gene-one enzyme hypothesis, 19
One-step mutants, 81, 88
Ontogenic theory, 35–36
O/R potential, 156, 158, 208
Oxygen, influence on radiation effects, 79

P

Pantothenic acid, 162
Papillae, 100, 112, 146
Paramecium, 21–25, 127, 211, 228
Pasteurella pestis, 46, 134
  P. tularensis, 106–107, 118–119, 123, 153, 158, 172
Pathogenesis, 218–222
Pathogenic bacteria, 104, 109, 114, 118, 121, 132, 138, 144, 165–166, 175, 218–222
Penicillin,
  and L-forms, 47
  effect on cell division, 41
  effect on colonial morphology, 113
Penicillin, effect on population changes, 158–159
  isolation of auxotrophs with, **93–94,** 97, 128–129, 212
  resistance, 63, **82–83,** 205–206, 222
  synergistic effects with iodoacetate, 191
Penicillinase, 183
Permeability, 87, 102
Peroxides, 69, 90, 135
pH, 103, 144, 158
Phage, 134, 144, 158, 192, 213–215, 228
  and L-forms, 47
  resistance to, **49–57,** 63, 66, 71, 73–74, 81, 111, 113, 133–136, 143, 164, 187, 195
  spontaneous vs. induced mutants resistant to, 69–70
Phagocytosis, 122, 166
Phase variation, 60, 63, 65, **114, 117–118,** 120, 140–142
Phases, 36
Phenocopy, **3,** 113
Phenomic lag, 13, 64–65, **73–75,** 77, 79, 196
Phenotype, **2–3,** 15, 17, 30, 61, 75, 101, 112–113, 116, 120–121, 138, 155, 176–179, 183, 196, 224, 227
Photodynamic effects, 69–70
Photoreactivation, 79
Phytomonas stewartii, 63, 103, 126, 186
  Ph. tumefaciens, 224
Pigmentation, 2, 63, 67, 101, **103,** 134, 145, 154
Plants, 10, 20
Plastids, 20
Pleiotropic genes, **16,** 129
Pneumococcus, 109, 191, 198–208, 210, 214, 222, 224
Poisson distribution, 53
Polyauxotrophic strains, 95
Polyploidy, **9,** 13, 46
Polysaccharides, 102, 115, 122, 134–135, 198–206, 208, 222
Population changes, **27–29,** 31–32, 34, 109, 126, **138–183**
  and inherent differences in metabolism, 159
  direction of, 155
  due to inhibitory effects of parent type 163–164

Population changes, effect of amino acids *in vivo* on, 168–170
effect of metabolites on, 146, **151–158,** 167–168
effect of method of cultivation on, 156–157, 163
effect of serum on, 157–158
effect of source of N on, 155–156
in liquid media, 148–164
in vivo, 165–170, 218–221
not due to mutation, 176–183
on solid media, 145–148
rate of, 150
role of metabolites in, 146, 151–154
Population dynamics, 64, 100, **138–164**
Population pressure, 149
Position effect, 15
Precursor, 16–17, 19, 87, 97–99, 135
Primary isolation, 109, **174–175,** 223
Progeny, 2–3, 23, 66, 138, 173–174, 193, 198, 224
of single cell, 48
of multinucleate cell, 77
Prophase, 5–6, 43–44
Proteus, 223
Prototrophs, **91,** 143, 187, 189, 193–194, 212–213, 226
Pseudomonas, 63, 223
Ps. putrefaciens, 180
Purines, 69, 95, 131–132, 164, 181, 201
Pyrimidines, 95, 201, 227
Pyrophosphate, 157, 161, 208

R

R type. See ROUGH TYPE.
Rabbit, 166
Radiation resistance, 41, 54, **88–90,** 110
Random isolation, 91
Rate of
biosynthetic processes, 19, 30
cell division, 41
establishment of mutants, 67, 117, 150
growth, 28, 41, 111, 117, 139–143, 145, 149–150
multiplication *in vivo*, 128, 131
mutation, 12, 14, 49, **62–67,** 69, 82, 101, 103, 115, 124–126, 139–142, 150, 169
population changes, **150,** 156, 159
Rate of recombination, 188, 193–194
Recessive genes, **9–14,** 42, 75, 195
Recombination, 100–101, 103, 180 **184–196,** 221, 225
as analytical tool, 195–196
frequency of, 188, 193–194
in *E. coli*, 186–194
mechanism of, 192–194
prevalence among different bacteria, 194
vs. transformation, 191–192
Reconstruction experiments, 164
Reduction division. See MEIOSIS.
Relapse, 219
Replica plating, **55–57,** 92, 95, 112
Reproduction, 46–48
asexual, 6 8, 21, 32, **46**
sexual, 5–8, 12, 20–21, 28, **46,** 127, 184, 186, 188–189, 194, 211
vegetative, 46
Reproductive error, 67
Residual growth, **65–66,** 144, 194
Resistance, 157, 167, 218–219
species differences in, 218
Resistance to
alanine, 120, 153–155, 167–168
aureomycin, 82, 86, 88
chloramphenicol, 83
crystal violet, 90
isonicotinic acid hydrazide, 82–83
neomycin, 83
nitrogen mustard, 90
penicillin, 63, **82–83,** 205–206, 222
peroxide, 90, 135
phage, **49–57,** 63, 66, 71, 73–74, 81, 111, 113, 133–136, 143, 164, 187, 195
potassium tellurite, 90
proflavine, 90
radiation, 41, 54, **88–90,** 110
safranin, 90
streptomycin, 29, 63, **81–82,** 88, 110–111, 155, 164, 187, 189, 191, 194–195
Terramycin, 83
threonine, 63, 111, 169–170
Resting cells, 27, 42, 179
mutations in, 67
Reversions, 17, 23, 77, **95–97,** 115, 117, **120–121,** 139, 164, 187, 202, 226–228
apparent, 96, 120, 155

Ribonucleic acid, 39
Rough type, 36, 63, **108–109,** 113–116, **118–120,** 124–125, 133, 135, 152–157, 159–162, 166, 168, 198–210, 218, 224
  cell division of, 47–48, 105
  nuclei of, 42

S

S type. See SMOOTH TYPE.
S′, 120, 155, 168
S°, 110, 125, 169–170
St, 110, 125, 169–170
Salmonella, 63, 99, 109–110, 115–118, 158, 194, 212–215, 221–223, 226
  S. gallinarum, 219–220
  S. typhimurium, 60, 63, 65, 111, 115, 117, 123, 125–126, 128–130, 134, 140, 153, 168–170, 212–215
  S. typhosa, 58, 63, 128–129, 131–132, 134
Screening procedures, 50, 62, 79, 81, 92, 110–111, 124, 128–129, 190
Seasonal variation, 131, 219, 221
Sectors, 76, 103, 146–147
Segregation, **11–12,** 20, 75, 146, 184–186, 188, 193, 196
Selection, **27–29,** 31, 33–34, 36–37, 138, **144–175**
  effects on determination of mutation rates, 65
  indirect, 55–58
  natural, 28, 225
  periodic, 141–143
Selective environment, 50, 52, 55, 62, 124, 138, 151, 158, 208, 218–220
  absolute, 144–145
  relative, 145–164
Selective value, **28–29,** 31, 109, 143, 146, 155, 161, 163 208, 224, 226–228
  determination of, 164
Serine, 97
Serratia marcescens, 63, 67, 103, 134, 140, 154
Serum effect, 157–158, 167
Serum factors, 157–158, 208
Sexual reproduction, 5–8, 12, 20–21, 28, **46,** 127, 184, 186, 188–189, 194, 211
Shigella dysenteriae, 63, 105, 134, 154, 226
  Shigella paradysenteriae, 208
  Sh. sonnei, 134
Small colony mutant, 25, 108, 126, 158
Smooth type, 36, 63, **108–109,** 113–116, **118–120,** 124–125, 132, 148, 150, 152–157, 159–162, 166, 168, 198–208, 218, 224
  cell division of, 47–48, 105
  nuclei of, 42
Species, 29, **223–226**
Spindle fibers, **5–6,** 43–44
Spontaneous changes, 12, 29, 49–50, 53, 61, 69, 126
  vs. induced mutants, 69
Spores, 40, 42, 46, 133
Staining, 39–40, 45
Staphylococcus, 45, 48, 63, 158, 223
Stepwise increase in resistance, **83,** 206
Stock cultures, maintenance of, 97, 148, **170–172**
Streptobacillus moniliformis, 47
Streptococcus, 48, 105, 134–135, 224
  Str. viridans, 224
Streptomycin-dependence, 63, 65, 73, **82,** 110, 155, 195, 226
Streptomycin-resistance, 29, 63, **81–82,** 88, 110–111, 155, 164, 187, 189, 191, 194–195
  isolation of mutants with, 57, 81
  origin of, 52, 56
  rate of mutation to, 49, 63
Substrate, 26–27, 101, 178–183, 217
Sulfonamides, 63, 158, 162
Suppressor genes, **17,** 96, 121, 136
Surface active agents, 113
Surface components, 104, 109, 115, 122, 131
Survival curves, 88
Survival value of mutants, 49, 155, 161 226
Susceptibility, 157, 167–168, 218–219
Symbols, 10, 114, 120, 187, 198
Synergistic effects, **131–132,** 191
Syntrophism, **97–98,** 131–132, 221

T

Taxonomy, 114, **223–225**
Telophase, **5–6,** 43–44
Temperature, 23, 127, 158, 171
  effect on mutation, 67, 126
Terramycin resistance, 83
Tetraploid, 9

Tetrazolium, 102
Theophylline, 80
Therapy, 87–88, 217, 221, 226
Thiamine, 227
Thiazole, 227
Threonine resistance, 63, 111, 169–170
Toxin, 122, 134
Trace metals, 157
Training, 58, 96
Transduction, 212–215, 222, 225
  vs. recombination, 213
Transformation, 100, **197–212,** 215, 222, 225
  allogenic, **203,** 222
  and epidemiology, 222
  autogenic, 203
  by lysates, 222
  conditions required for, 207–209
  in *Paramecium*, 23
  mechanism of, 211–212
  of colonial morphology, 206–207
  of drug resistance, 205–206, 222
  of pneumococcal capsular types, 198–204
  of protein specificity, 204–205
  reciprocal, 207
  sensitization in, 210
  time required for, 210–211
  vs. recombination, 191–192
Transforming principle (TP), 200–210, 222
Translocations, 15
Tribe, 223
Triploid, 9
Tryptophan, 58, 69–70, 97, 99, 181, 212
Tryptophan-independence, 58, 67, 212
  mutational origin of 58–60
Turbidity measurements, 60, 145, 148, **173–174**
Turbidostatic selector, 84
Type specificity, **115,** 135, 198, 208–210

U

U-tube, 192, 212–213
Ultraviolet irradiation, 14, 41–42, 69–72, 74, 76, 79, 88–89, 129, 146–147
Uninucleate, 41–42, 196

V

Vaccine, 171, 173
Valine, 98, 133, 136, 156
Viability, 149–151
Virulence, 104, 109, 111, **121–124,** 133–136, 153, 165–170, 204, 215–222
  antigenic characteristics and, **123,** 134
  colonial morphology and, **123–124,** 134
  detection of, 106
  development of, 99
  inherent properties affecting, 121–122
  mutational origin of changes in, 123
  of auxotrophic mutants, 127–133
Virus, 21, 215
Vitamins, 95, 99, 187, 216

X

X-rays, 14, 69–71, 75, 79, 129
Xylose fermentation, 187

Y

Yeast, 20, 25–27, 87, 127

Z

Zygote, **6,** 188

CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE
LIBRARY
MYSORE.

C. F. T. R. I. LIBRARY, MYSORE.

Acc No. 2921

Call No. ~~GT:6 J3~~ GV,21;6 N53

Please return this publication on or before the last DUE DATE stamped below to avoid incurring overdue charges.

Due Date	Return Date	Due Date	Return Date
(1) Reserved for Miss. K.R. Vijayalakshmi			6/7/71
23.7.71	22/7	~~Ref. 420~~	
27.8.71	27/8	8.11.76	4/11
11.9.71	10/9	26.1.77	27.1.77
1.10.71	5/10	11.2.77	10.2.77
~~3.F~~		25.2.77	25.2.77
11.1.72	4.1.72	12/3/77	10.3.77
7 JUL 1972	27 JUN 1972	25.3.77	28/3
26 NOV 1972	26.11.72	3.11.77	3/11
17.8.74	12.8.74	18.9.90	3/9 PTO
12.10.76	11-10-76		

CFTRI-MYSORE
2921
Bacterial geneti..

RAUN
953